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Pathogenicity and Germination of the Entomopathogenic Fungi *Beauveria bassiana* and *Purpureocillium lilacinum* under Different Temperature Changes against *Spodoptera littoralis* Larvae

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Authors' contributions

This work was carried out in collaboration between both authors. Authors SMIAEK and SAI designed the study. SMIAEK performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SMIAEK and SAI managed the analyses of the study. Author SMIAEK managed the literature searches. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Since thermal stress influences the whole infection pathway, temperature is a critical factor in determining the efficiency of entomopathogenic fungi in microbial pest management. This study aimed to determine how temperature changes affect the germination rate of two isolating entomopathogenic fungi. In addition, we looked at how different entomopathogenic fungi performed against *Spodoptera littoralis* second-instar larvae after being incubated at various temperatures. Two locally isolated entomopathogenic fungi were identified morphologically as *Beauveria bassiana* and *Purpureocillium lilacinum*. Both fungi were incubated at 15, 20, 25, 30, 35, and 40° C to evaluate their germination and pathogenicity. Results showed that both fungi could germinate effectively at 20-35° C. Results also showed that *P. lilacinum* can germinate at all tested temperatures. No germination was obtained at 40° C for both fungi. *B. bassiana* was more pathogenic against *S. littoralis* larvae than *P. lilacinum*. The results showed that the isolated fungi can be developed as potential biopesticides against *S. littoralis* under different temperatures, although proper formulation is still required.

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Keywords: Spodoptera littoralis; entomopathogenic fungi; temperature; germination; pathogenicity.

ABBREVIATIONS

ANOVA : Analysis of variance
EPF : Entomopathogenic fungi
PDA : Potato Dextrose Agar
SDA : Sabourad Dextrose Agar
SDYA : Sabourad Dextrose Yeast Agar
SPSS : Statistical Package for Social Sciences

1. INTRODUCTION

Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae), commonly known as the Egyptian cotton leafworm, is a highly polyphagous and destructive pest that poses a significant threat to a variety of economically important crops worldwide [1–4]. Larvae of Lepidopteran insects are a matter of worldwide phytosanitary concern because of their capacity to infest various plant structures, resulting in detrimental effects on crop quality and significant economic losses [5]. An increased population can lead to leaf skeletonization, resulting in crop loss, damage to the shoot system, and significant economic implications [6]. The widespread utilization of traditional insecticides in controlling *S. littoralis* has led to resistance against prominent pesticide categories [6]. It can exert adverse effects on the surrounding ecosystem [7]. In light of the rise of resistance and environmental risks, it is imperative to explore alternative methods of pest control that offer greater cost-effectiveness and sustainability compared to traditional insecticides [8]. Finding safe substituents for these compounds is a crucial need. Bioinsecticides, such as compounds derived from bacteria, fungi, and viruses, should be considered more [9–11]. These groups have distinctive modes of action [11], and their properties may vary significantly from those of the conventional compounds to which producers are accustomed. Entomopathogenic fungi (EPF) can infect and kill arthropods. Even though they are primarily isolated from arthropod remains, their natural habitat is soil [12]. The primary function of EPF in the environment is insect population control. The literature provides information regarding the infectious properties of EPF, the infectious process [13,14], and the use of these microorganisms as biopesticides in commercial preparations [15]. EPF, with a focus on hypocrealean ascomycetes, are unique among entomopathogenic microorganisms due to their mode of action through the cuticle, which gives them a significant advantage in integrated pest management; conidia of EPF adhere to the cuticle, germinate, penetrate the host (without the need for ingestion), grow inside the hemocoel, and ultimately cause the death of the host due to nutrient deficiencies [16]. These fungi can control a wide variety of arthropod pest species, including locusts and grasshoppers, soil-dwelling insects, penetrating and sucking insects, mites, stored-grain pests, several forestry pests, and invasive, medicinal, and veterinary pests [17]. Temperature is a critical determinant of the efficacy of EPF in microbial pest control, as thermal stress influences the entire infection pathway [18]. In general, EPFs are mesophilic microorganisms with growth potential between 10 and 40°C, and optimal growth between 25 and 35°C, and the geographical origin of an entomopathogenic fungal strain is a significant factor in determining tolerance to high or low temperature[18–23]. Entomopathogenic fungi (EPF) such as *Beauveria* and *Metarhizium* development is significantly constrained by extremely high and low temperatures [24,25]. A recent analysis by Tong and Feng [26] has examined the phenotypic and molecular aspects of heat tolerance in entomopathogenic fungi (EPF). This review presents substantial data indicating that *Beauveria* and *Metarhizium*, two often studied EPFs, exhibit a heightened sensitivity to high temperatures exceeding their upper thermal limits (32–35°C) during the summer. The effectiveness of mycoinsecticide field applications is limited by heat tolerance in Mediterranean climatic conditions [26]. In light of these facts, the present investigation aims to assess the direct effect of temperature fluctuations on the germination ability of two isolated entomopathogenic fungi. Furthermore, the virulence of tested entomopathogenic fungi after growing under different temperatures against the 2nd instar larvae of *S. littoralis* was investigated.

2. MATERIALS AND METHODS

1. 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 [27].

2. Selective medium for isolation of entomopathogenic fungi

ollected 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 [28]. 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.

3. Fungal characteristics and morphological identification

Each colony's 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 [29]. 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 [30].

4. Effect of temperature fluctuations on fungal growth

To determine the influence of temperature on germination, 0.1 ml of conidial suspension containing 1×10^8 conidia ml^{-1} was spread-plated on SDA plates in Petri dishes. On each plate, sterile microscope coverslips were inserted. Inoculated dishes were sealed with Parafilm M and incubated in total darkness at 15, 20, 25, 30, and 35° C and 95±5% R. H. Twenty-four hours after inoculation, 1 ml of formaldehyde (0.5%) was added to each plate to prevent germination. Using a hemocytometer, the germination percentage was calculated by counting 100 spores per plate under a magnification of X40. Each plate was a replicate, with four replicates per treatment [31].

5. Effect of temperature on fungal virulence

Spores were scraped from the surface of cultures and utilized to make spore suspensions (distilled water with 0.1% Tween 80). By immersing newly molted 2nd instar larvae in the prepared solutions for 10 seconds, all isolated fungal species were evaluated at a constant concentration of 1×10^8 conidia ml^{-1} in 0.1% Tween 80. The control group was treated with sterile distilled water with 0.1% Tween 80. Following treatment, treatment was replicated four times, and each replicate of 20 insects was put in a rearing container and incubated for 48 hours at 25°±2 C and 95±5% R. H. Larval mortality was recorded daily 48h post-treatment for 14 days till pupation. It was corrected according to Abbott's formula [32,33]. Dead larvae were placed in a petri dish lined with moistened filter paper. Mortality due to fungal infection was confirmed by observation of hyphal and spore growth on the surface of the dead insect's body (Fig. 1) [31].

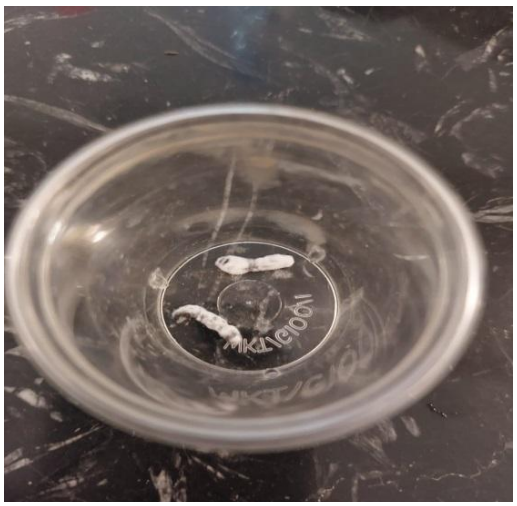


Fig. 1. Sporulation of entomopathogenic fungi on larval cadavers confirming the fungal infection

6. Statistical analysis

Germination data were subjected to analysis of variance (ANOVA) for a completely randomized design using SPSS 22.0 (Statistical Package for Social Sciences, USA) version 22.0.0 software. The results were presented as the mean and

standard deviation. Significant differences between means were identified at the $P < 0.05$ level [33]. After culturing at different temperatures, the pathogenicity of isolated fungi was analyzed using ANOVA, and results were expressed as the mean and standard deviation. The variance ratio (Eta^2) was measured to assess the effect of temperature on fungal germination and virulence using SPSS 22.0 software.

3. RESULTS

1. Identification and morphological characterization of isolated fungi

Two entomopathogenic fungi were identified based on the morphological features of isolated fungi. *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. 2).

In addition, the second fungus was identified as *Purpureocillium lilacinum* ((Thom) Luangsa-ard, Hou-braken, Hywel-Jones & Samson) (Hypocreales: Ophiocordycipitaceae). Conidia were ovoid to spindle-shaped, single-celled, chain-like spores with a 3.1-4.0 µm diameter. The formation of white colonies with white outlines characterized early phases. The growth eventually obtained purple tints. The colonies were purple, spherical, and inflated after seven days. The surface had no secretion, and the consistency resembled cotton (Fig. 3).

a

b

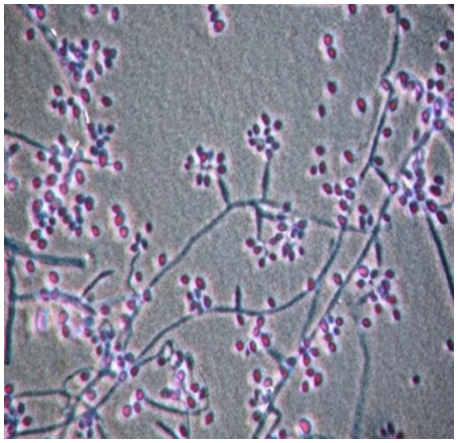
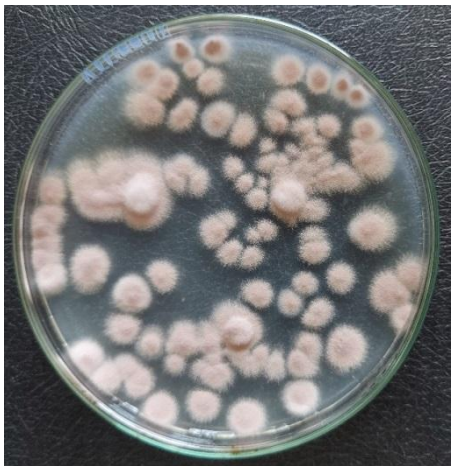


Fig. 2. Culture characteristics of the 14-day colony (a) and microscopic examination showing the conidiophore and conidia (b) for *Beauveria bassiana*

a



b

c

Fig. 3. Culture characteristics of the 7-day colony (a), 14-day colony (b), and microscopic examination showing the conidiophore and conidia (c) for *Purpureocillium lilacinum*

a

b

c

d

e

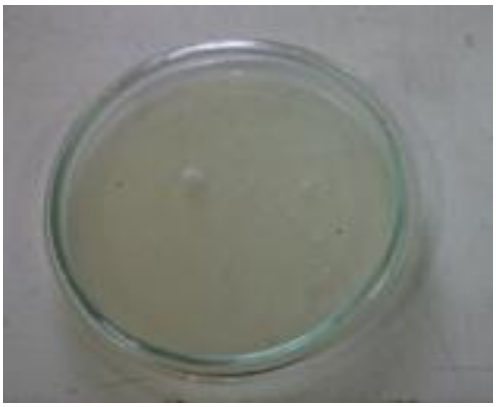


Fig. 4. *Beauveria bassiana* fungal germination at different temperatures for 14-day; (a) 15°C, (b) 20°C,(c) 25°C,(d) 30°C, and (e) 30°C

2. Effect of temperature on isolated fungi germination

The impact of temperature on fungal germination of *B. bassiana* and *P. lilacinum* is presented in Table 1 regarding conidial count. The morphological appearance of *B. bassiana* colony germination under different temperatures is shown in Fig. 4. It was noticed that the optimal fungal germination was obtained at 20, 25, and 30° C. The fungal growth was almost observed at 35°C, but no fungal growth at 40°C. Furthermore, the conidial count of *B. bassiana* was lowered at low (15°C) and high (35°C) temperatures (2.9×10^4 conidia ml^{-1} and 0.3×10^2 conidia ml^{-1}, respectively). The highest conidial counts were observed at 20 and 25°C (4.3×10^9 and 4.5×10^9 conidia ml^{-1}, respectively).

The morphological appearance of *P. lilacinum* colony germination under different temperatures is shown in Fig. 5. *P. lilacinum* can combat high temperatures, and high fungal growth was obtained at all temperatures. There was no fungal growth at 40°C. Furthermore, the conidial count of *P. lilacinum* was directly related to temperature; as the temperature raised, the conidial count increased. The conidial count rarely declined at 30 and 35°C (Table 1).

a

b

c

d

e

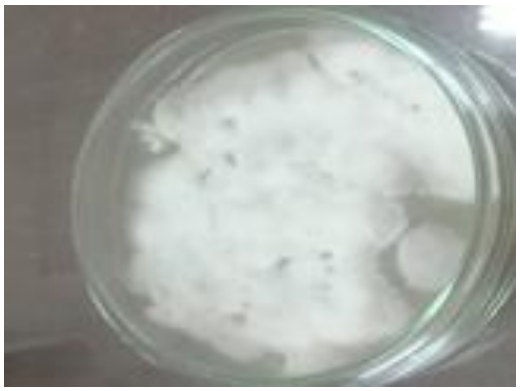


Fig. 5. *P. lilacinum* fungal germination at different temperatures for 14-day; (a) 15°C, (b) 20°C,(c) 25°C,(d) 30°C, and (e) 30°C

Table 1. Fungal conidial count of *Beauveria bassiana* and *Purpureocillium lilacinum* in different temperatures

Treatment	Conidial count at different temperatures (Mean ± S.E.) (Conidia ml^{-1})				
	15°	20°	25°	30°	35°
Beauveria bassiana	$2.9\times10^{4}\pm0.003$*	$4.3\times10^{9}\pm0.03$*	$4.5\times10^{9}\pm0.02$*	$2.7\times10^{9}\pm0.003$*	$0.3\times10^{2}\pm0.03$*
Purpureocillium lilacinum	$3.1\times10^{6}\pm0.002$*	$5.8\times10^{9}\pm0.01$*	$8.3\times10^{9}\pm0.002$*	$1.7\times10^{10}\pm0.00$*	$2.4\times10^{8}\pm0.001$*

*: Significant at P ≤ 0.05

Table 2. Virulence of tested entomopathogens under temperature intervals concerning larval mortality

Treatment	Larval mortality at different temperatures (Mean ± S.E.)					P-value
	15°	20°	25°	30°	35°	
Beauveria bassiana	8.0±0.58*	16.0±1.15*	17.0±0.58*	13.0±0.58*	5.0±0.58*	0.00
Purpureocillium lilacinum	4.0±0.57*	9.0±0.57*	7.0±0.57*	6.0±0.00*	4.0±0.54*	0.00
Control	0	0	0	0	0	

: Significant at P ≤ 0.05

Measurement of the association of fungal germination and temperature showed a high association in both *B. bassiana* and *P. lilacinum* (Eta^2= 1.00 for both fungi).

3. Effect of temperature on fungal virulence

The fungal virulence of *B. bassiana* and *P. lilacinum* regarding their pathogenicity against the 2nd instar larvae of *S. littoralis* is shown in Table 2. Results showed that *B. bassiana* has high larvicidal activity than *P. lilacinum*. In addition, the temperature affected the virulence of both fungi, as confirmed by larval mortality. Both fungi caused low larval mortality when fungal isolates were incubated at 15 and 35° C. High larval mortality was acquired when larvae were treated with fungi incubated at 20, 25, and 30° C for *B. bassiana* and at 20°C for *P. lilacinum*. Moreover, *B. bassiana* was more infective than *P. lilacinum* against the 2nd instar larvae of *S. littoralis*. Measurement of the association of fungal virulence and temperature showed that *B. bassiana* virulence against the 2nd instar larvae was temperature dependant (Eta^2= 0.976) regarding the untreated larvae. On the other hand, *P. lilacinum* virulence against the 2nd instar larvae was temperature dependant (Eta^2= 0.933) considering the control.

4. DISCUSSION

Temperature is a crucial determinant of the efficacy of EPF in microbial pest control, as thermal stress affects the entire infection pathway [18]. The role of temperature in influencing biological processes is crucial, and its association with geographic location results in regional variability that must be considered when dealing with live organisms. This feature becomes particularly pertinent when considering the significant environmental strain linked to

global warming and the escalating harsh circumstances that are increasingly encountered. In this context, the present investigation aimed to assess the effect of temperature fluctuations on the germination and virulence of two local entomopathogenic fungi, *B. bassiana,* and *P. lilacinum*. Results revealed that both fungi could germinate effectively under optimum temperatures ranging from 20-30°C. Furthermore, results showed that *P. lilacinum* was heat tolerant compared to *B. bassiana*. In addition, both fungi failed to germinate at 40°C. In general, EPFs are mesophilic microorganisms with a growth potential between 10 and 40° C and an optimal growth ranging from 25 to 35° C, and a strain's geographical origin is a major factor in determining its tolerance to high or low temperature [21,23,34]. *B. bassiana* was more toxic against the 2nd instar larvae of *S. littoralis*. De Croos et al. [35] found that proteins in cold-active fungi strains differed from those in a non-cold-active strain when cultivated at 8 °C compared to 25 °C. These differently produced proteins may affect the pathogenicity of fungi. Thermotolerance-related genes may be present in certain strains, influencing their pathogenicity [36]. The development of entomopathogenic fungi (EPF) such as *Beauveria* is significantly constrained by high and low temperatures [24,25]. A recent analysis by Tong and Feng [26] has examined the phenotypic and molecular aspects of heat tolerance in EPF. The research presented extensive data indicating that *Beauveria* and *Metarhizium* exhibited high sensitivity to elevated temperatures that exceeded their top thermal limits (32-35 °C) during the summer months. The effectiveness of mycoinsecticide field applications is limited by heat tolerance in Mediterranean climatic conditions [26]. Results also show that *B. bassiana* was more toxic to the 2nd instar larvae of *S. littoralis* than *P. lilacinum*. Previous findings revealed that *P. lilacinum* and *B. bassiana* could be isolated from infected insect pests and potentially control many insects [37–44]. According to previous reports, *P. lilacinum* also showed varied insecticidal activities against insects [42,44–46].

5. CONCLUSION

Locally isolated *B. bassiana* and *P. lilacinum* showed larvicidal activity against the *S. littoralis* larvae. Besides, heat tolerance capabilities make them good alternatives to conventional insecticides to be used under hot weather. Accordingly, both fungi can be developed as potential biopesticides against *S. littoralis,* although the development of proper formulation is still required.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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