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Review Article

Temperature effects on the virulence of entomopathogenic fungi to manage the bronze bug *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

Efeitos da temperatura sobre a virulência de fungos entomopatogênicos para o manejo do percevejo-bronzeado *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

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Abstract

Thaumastocoris peregrinus Carpintero & Dellapé (Hemiptera: Thaumastocoridae), a sap-sucking pest native to Australia, has severely impacted eucalyptus plantations in Brazil since 2008. Due to environmental concerns and resistance risks, chemical control is restricted in forest systems, prompting the search for sustainable alternatives. Entomopathogenic fungi (EPF) offer promising potential for integrated pest management (IPM) strategies. This study evaluated the virulence and thermal tolerance of EPF isolates against *T. peregrinus* through a multi-phase bioassay. Initially, eleven fungal isolates were screened for pathogenicity against adults. Based on mortality rates, three isolates *Beauveria bassiana* (IBCB227), *Metarhizium anisopliae* (IBCB425), and *Cordyceps farinosa* (IBCB220) were selected for further testing at constant temperatures (20, 25, and 30 °C). Subsequently, lethal concentration (LC_{50}) and lethal time (LT_{50}) were determined for the two most virulent isolates (IBCB227 and IBCB425) in both nymphs and adults. IBCB425 demonstrated high efficacy across all temperatures, while IBCB227 and IBCB220 were less effective at 20 °C. LC_{50} values were 1.10×10^7 and 2.10×10^6 conidia/mL for IBCB227 and IBCB425, respectively, based on mortality recorded six days post-application. LT_{50} was calculated using the highest concentration (1.0×10^8 conidia/mL), with IBCB425 inducing faster mortality (3.75 days) than IBCB227 (4.43 days). Post-mortem sporulation confirmed fungal infection in >90% of cadavers. These findings highlight the virulence and temperature resilience of *M. anisopliae* IBCB425 and *B. bassiana* IBCB227, supporting their potential as effective biological control agents for *T. peregrinus* in eucalyptus plantations under variable environmental conditions.

Keywords: bronze bug, entomopathogenic fungus, isolate selection, microbial control, sustainable forest.

Resumo

Thaumastocoris peregrinus Carpintero & Dellapé (Hemiptera: Thaumastocoridae), um inseto sugador de seiva nativo da Austrália, tem causado severos impactos em plantações de eucalipto no Brasil desde 2008. Devido a preocupações ambientais e ao risco de desenvolvimento de resistência, o controle químico é restrito em sistemas florestais, o que impulsiona a busca por alternativas sustentáveis. Fungos entomopatogênicos (FEP) apresentam potencial promissor para estratégias de manejo integrado de pragas (MIP). Este estudo avaliou a virulência e a tolerância térmica de isolados de FEP contra *T. peregrinus* por meio de um bioensaio em múltiplas fases. Inicialmente, onze isolados fúngicos foram avaliados quanto à patogenicidade em adultos. Com base nas taxas de mortalidade, três isolados — *Beauveria bassiana* (IBCB227), *Metarhizium anisopliae* (IBCB425) e *Cordyceps farinosa* (IBCB220) — foram selecionados para testes adicionais em temperaturas constantes (20, 25 e 30 °C). Posteriormente, foram determinadas a concentração letal (CL_{50}) e o tempo letal (TL_{50}) para os dois isolados mais virulentos (IBCB227 e IBCB425), tanto em ninfas quanto em adultos. O isolado IBCB425 apresentou alta eficácia em todas as temperaturas, enquanto IBCB227 e IBCB220 foram menos eficientes a 20 °C. Os valores de CL_{50} foram de $1{,}10 \times 10^7$ e $2{,}10 \times 10^6$ conídios/mL para IBCB227 e IBCB425, respectivamente, com base na mortalidade registrada seis dias após a aplicação. O TL_{50} foi calculado utilizando a maior concentração ($1{,}0 \times 10^8$ conídios/mL), sendo que IBCB425 induziu mortalidade mais rápida (3,75 dias) em comparação com IBCB227 (4,43 dias). A esporulação pós-morte confirmou a infecção fúngica em mais de 90% dos cadáveres. Esses resultados destacam a virulência

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e a resiliência térmica de *M. anisopliae* IBCB425 e *B. bassiana* IBCB227, reforçando seu potencial como agentes eficazes de controle biológico de *T. peregrinus* em plantações de eucalipto sob condições ambientais variáveis.

Palavras-chave: percevejo-bronzeado, fungos entomopatogênicos, seleção de isolados, controle microbiano, floresta sustentável.

1. Introduction

The bronze bug, *Thaumastocoris peregrinus* Carpintero & Dellapé, 2006 (Hemiptera: Thaumastocoridae), is a sap-sucking insect native to Australia that has been a serious pest in commercial eucalyptus plantations in Brazil since 2008 (Dias et al., 2014; Salibá et al., 2019). Its feeding causes the destruction of leaf tissues, leading to chlorosis, reduced photosynthesis, and premature leaf abscission, which significantly compromises tree growth and the productivity of planted forests (Oumar et al., 2013).

Thaumastocoris peregrinus has also been reported in other eucalyptus-growing countries, such as Argentina, Chile, South Africa, Italy, and Portugal, underscoring its invasive potential and the need for coordinated international control strategies (Jacobs and Neser, 2005). In Brazil, where eucalyptus is a cornerstone of the forestry economy, planted forests contribute to pulp and paper production, bioenergy, and carbon sequestration, reinforcing the importance of pest management in these ecosystems (ABRAF, 2023). Several studies have investigated the use of entomopathogenic fungi to manage *T. peregrinus* in Brazil (Santos et al., 2018; Tedesco et al., 2020; Velozo et al., 2023), but few have evaluated the combined effects of temperature, virulence, and developmental stage on fungal performance.

Infestation by *Thaumastocoris peregrinus* presents a challenge to the sustainable management of eucalyptus plantations, particularly because chemical insecticide use is restricted due to environmental concerns and the risk of selecting resistant insect populations (Wilcken et al., 2019; Bamisile et al., 2021). In this context, alternative strategies such as biological control using entomopathogenic fungi (EFs) have emerged as promising, sustainable approaches to reduce pest-related damage. In addition to laboratory selection, isolating EPF strains directly from naturally infected *T. peregrinus* or from the eucalyptus ecosystem (soil, leaves) is widely recognized as a strategy to enhance ecological fitness (Corallo et al., 2019; Mascarin et al., 2012; Lorencetti et al., 2017). However, the present study focused on pre-selected strains with established cultivation potential, aiming to assess virulence under ecologically relevant thermal conditions typical of Brazilian eucalyptus regions (Zimmermann, 2008; Fargues et al., 1997).

Entomopathogenic fungi (EPFs) effectively control sap-sucking pests such as hemipterans and coleopterans, offering several advantages over chemical pesticides, including specificity, safety to nontarget organisms, and environmental compatibility (Zimmermann, 2007; Dal Pogetto et al., 2011; Lorencetti et al., 2018; Mascarin Moura et al., 2018; Velozo et al., 2023). EPFs act mainly through conidial adhesion, cuticle penetration, and the release of toxic metabolites, leading to host mortality, while reducing the need for synthetic insecticide molecules in cultivated areas (Islam et al., 2021). Moreover, EPFs can be mass produced in vitro, formulated, stored, and applied as bioinsecticides in forest and agricultural environments (Velozo et al., 2023).

Although the egg parasitoid *Cleruchoides noackae* Lin & Huber (Hymenoptera: Mymaridae) was introduced from South Africa for the biological control of *T. peregrinus* in Brazil (Barbosa et al., 2017), its production is limited by the absence of alternative hosts, making it necessary to rear *T. peregrinus*, which increases production costs (Martínez et al., 2018). This highlights the importance of developing and optimizing additional biological control strategies, such as the use of entomopathogenic fungi or native predators such as *Supputius cincticeps* Stål (Hemiptera: Pentatomidae) (Souza et al., 2012), for effective and integrated management of this pest.

Temperature and humidity are crucial abiotic factors that directly influence the infection dynamics and performance of entomopathogenic fungi (EPF). These factors affect key stages of the fungal life cycle, including spore germination, hyphal growth, host cuticle penetration, and sporulation, ultimately determining the efficacy of fungal-based pest control in the field (Fargues et al., 1997; Inglis et al., 2001). High temperatures can accelerate fungal growth within the host but may reduce viability if exceeding optimal thresholds, while low temperatures may suppress or delay fungal germination and infection (Zimmermann, 2008).

Similarly, relative humidity plays a pivotal role in conidial adhesion and cuticle penetration, with low humidity conditions negatively impacting EPF infection success. Given the environmental variability in eucalyptus plantations across Brazil particularly fluctuations in temperature and moisture identifying isolates that are both virulent and thermotolerant is fundamental for achieving reliable biological control. In this context, *T. peregrinus* has emerged as a major pest, and various studies have demonstrated its susceptibility to different EPF species (Lorencetti et al., 2018; Santos et al., 2018; Velozo et al., 2023).

This study builds upon previous field evaluations (Wilcken et al., 2019) by conducting a detailed laboratory-based assessment of virulence and thermal tolerance of eleven entomopathogenic fungal isolates. These controlled assays allow us to identify promising isolates with superior performance across temperature ranges and insect developmental stages, thereby supporting formulation and application strategies under diverse climatic conditions.

This study aimed to evaluate the pathogenicity of different isolates of entomopathogenic fungi against *T. peregrinus* at constant temperatures and to determine the median lethal concentrations (LC_{50}) of the most effective isolates. By identifying isolates that combine high virulence and temperature resilience, this research contributes to the development of sustainable biopesticide and integrated pest management (IPM) strategies for eucalyptus plantations.

2. Materials and Methods

2.1. Location

The experiments were conducted at the Laboratory of Biological Control of Forest Pests (LCBPF) and at the Application Technology Laboratory of the Department of Plant Protection (DPV) of the Faculty of Agronomic Sciences (FCA) at São Paulo State University (UNESP) in Botucatu, São Paulo state, Brazil.

2.2. Insect rearing

The colony of *T. peregrinus* was established using eggs from the stock culture at the entomology laboratory of Embrapa Florestas in Colombo, Paraná state, Brazil. Insects were maintained in a climate-controlled room at 24 ± 2°C, a relative humidity of 60 ± 10%, and a 12-hour photoperiod (Barbosa et al., 2017). Nymphs hatched from these eggs were kept on branches of *Eucalyptus urophylla* var. platyphylla, clone 433 (Myrtaceae), in 500 mL Erlenmeyer flasks with water and placed in rectangular plastic trays (40 cm long × 35 cm wide × 8 cm high). Adults and nymphs from the laboratory rearing system were used in the pathogenic bioassays.

2.3. Selection of entomopathogenic fungus isolates

Isolates of the entomopathogenic fungi *Beauveria bassiana (Bals. Criv.)* Vuill. (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales: Clavicipitaceae), *Cordyceps farinosa* (Holmsk.) Fr. (Hypocreales: Cordycipitaceae), *Cordyceps fumosorosea* Wize (Hypocreales: Cordycipitaceae), and *Sporothrix insectorum* Hoog & H.C. Evans (Ophiostomatales: Ophiostomataceae) (Table 1) were obtained from the "Odemar Cardim Abreu" Collection of Entomopathogenic Microorganisms at the Biological Institute of Campinas, São Paulo state, Brazil, which were all collected within this state.

The initial inoculum of each isolate was obtained through propagation in Petri dishes with potato dextrose agar (PDA) (42 g/L [Kasvi, Italy]) at 25 ± 1°C in the dark.

The bioassay was initiated with infective structures of the fungi (conidia) collected by gently scraping the surface with a Drigalski loop and growing for 14 days on PDA medium. The material obtained was suspended in Tween 80® [Sigma, Germany] (0.1%). Prior to the bioassays, conidial viability was assessed by germination tests. A 100-µL aliquot of each conidial suspension was spread onto PDA medium and incubated at 25 ± 1°C for 24 h. Germination was evaluated under a light microscope by counting at least 100 conidia, and isolates presenting viability greater than 90% were used in the experiments.

The concentration of the conidial suspension was adjusted to 1.0×10^8 conidia/mL using a hemocytometer, and conidia were counted under an optical microscope [Carl Zeiss Microscopy GmbH, Germany]. Ten adult individuals of *T. peregrinus* were used per replicate in the bioassay to screen fungal isolates for pathogenicity from the laboratory were placed in modified Petri dishes (60 × 20 mm) with lids 20 mm in diameter and covered with voile fabric for gas exchange. Hydroretentive gel® [Hydroplan-EB, Empresa de Base & Distribuidora Ltda, Brazil] (1 g/400 mL) was applied underneath the *E. urophylla* var. *platyphylla*, clone 433, leaf with an approximate area of 6 cm^2 used per Petri dish. This gel reduces water loss from the leaf and prevents insects from escaping. Two milliliters of conidial suspension were sprayed onto each group of 10 adult *T. peregrinus* per Petri dish via a Potter Tower, whereas the control group received only Tween 80® (0.1%). These Petri dishes were kept in Biochemical Oxygen Demand (B.O.D.) incubators at 25.0 ± 1.0°C, a relative humidity of 83.0 ± 2.0%, and a 12-hour photoperiod. The experiment was conducted in triplicate, each repetition performed on a different day with freshly prepared suspensions and new insect cohorts. For each isolate–temperature combination, five replicates (Petri dishes) were used per repetition, totaling 15 replicates and 150 insects per treatment. Each replication (Petri dish) was observed daily, and the dead

Table 1. Isolate codes (Code), species, source and host orders (Ord.) of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, *Cordyceps* sp., *Cordyceps farinosa*, *Cordyceps fumosorosea*, and *Sporothrix insectorum* used.

Codes	Specfies	Hosts	Ordens
IBCB227	*Beauveria bassiana*	*Spodoptera frugiperda*	Lepidoptera
IBCB226	*Beauveria bassiana*	*Bactris gasipae* (Plant)	-
IBCB66	*Beauveria bassiana*	*Hypothenemus hampei*	Coleoptera
IBCB617	*Beauveria bassiana*	*Myzus persicae*	Hemiptera
IBCB532	*Beauveria bassiana*	Larvae	Lepidoptera
IBCB425	*Metarhizium anisopliae*	Soil	-
IBCB348	*Metarhizium anisopliae*	*Mahanarva fimbriolata*	Hemiptera
IBCB375	*Cordyceps* sp.	Leafhopper	Hemiptera
IBCB220	*Cordyceps farinosa*	Soil	-
IBCB638	*Cordyceps fumosorosea*	*Bemisia tabaci*	Hemiptera
IBCB79	*Sporothrix insectorum*	*Leptopharsa hevea*	Hemiptera

insects were counted, removed, and transferred to humid chambers (insects were placed in sterile Petri dishes sealed with lids). Sterilized absorbent paper was placed at the bottom of the Petri dishes, which were previously moistened with autoclaved distilled water to stimulate fungal growth and confirm insect mortality. Bioassays were conducted until mortality stabilized, allowing the determination of LT_{50} (lethal time for 50% of the population) and the efficacy of fungal isolates.

All assays were independently repeated three times on separate dates, using freshly prepared suspensions for each replicate and new groups of *T. peregrinus* adults. Each replicate consisted of five Petri dishes with ten insects each (n = 50 insects per treatment per repetition), totaling 150 insects per treatment. Mortality was recorded daily for seven days, and cadavers were transferred to humid chambers to confirm fungal infection through sporulation.

2.4. Pathogenicity of Beauveria bassiana, Cordyceps farinosa, and Metarhizium anisopliae to bronze bugs at different temperatures

The preselected isolates of the entomopathogenic fungi *B. bassiana* (IBCB227), *Cordyceps farinosa* (IBCB220), and *M. anisopliae* (IBCB425) were used. Conidial suspensions were prepared and applied as previously described in the subsection "Selection of Entomopathogenic Fungus Isolates" at a concentration of 1.0×10^8 conidia/mL. Ten insects (replicates) were prepared in the same manner per modified Petri dish. Both nymphs and adults were tested separately under each temperature condition, using ten individuals per stage per replicate. Following inoculation, the replicates were maintained in B.O.D. incubators at temperatures of 20, 25, or 30°C with a relative humidity of 81.0 ± 2.0% and a 12-hour photoperiod. Three temperatures (20, 25, and 30°C) were selected because they represent the range commonly observed in Brazilian eucalyptus-growing regions where *T. peregrinus* occurs and where entomopathogenic fungi are applied under field conditions (Fargues et al., 1997; Zimmermann, 2008). Each replicate (Petri dish) was observed daily, and the dead insects were counted, removed, and transferred to humid chambers to stimulate fungal growth and confirm the cause of insect mortality. The inoculation experiment was conducted in a factorial arrangement consisting of three fungal isolates (*B. bassiana* IBCB227, *C. farinosa* IBCB220, and *M. anisopliae* IBCB425) and three temperatures (20, 25, and 30°C), with five replicates per treatment. Each replicate consisted of 10 insects, totaling 50 insects per treatment. The experiment was repeated three as independent blocks.

2.5. Lethal concentration (LC_{50}) and lethal time (LT_{50}) of selected isolates to Thaumastocoris peregrinus

The LC_{50} and LT_{50} values of *Metarhizium anisopliae* (IBCB425) and *Beauveria bassiana* (IBCB227) were determined using conidial suspensions prepared and applied as previously described. Separate bioassays were conducted for nymphs and adults of *Thaumastocoris peregrinus*, with ten individuals per stage per replicate in modified Petri dishes. The treatments included isolates applied at concentrations of 1.0×10^4, 10^5, 10^6, 10^7, and 10^8 conidia/mL, with the replications kept in B.O.D. incubators at 25°C, a relative humidity of 83.0 ± 2.0% and a 12-hour photoperiod. Each replication (Petri dish) was observed daily, and the dead insects were counted, removed, and transferred to humid chambers to stimulate fungal growth and to confirm the cause of insect mortality. Isolates with viability above 90% were used. The treatments involved two fungal species (level 1) and five concentrations (level 2) with five replications, totaling 50 insects per treatment/level, and the experiments were conducted in two independent blocks. Isolates from different fungal species were selected on the basis of the total number of insects that died and the mortality period.

2.6. Statistical analysis

Statistical analyses were performed to estimate lethal concentration (LC_{50}) and lethal time (LT_{50}) values with 95% confidence intervals using Probit regression models, appropriate for binomial response data (Nelder and Wedderburn, 1972). Each model was fitted separately for each fungal isolate and insect developmental stage combination. LC_{50} estimates were based on cumulative mortality recorded at day 6 post-inoculation, the point at which mortality plateaued. LT_{50} estimates were derived from data collected using the highest concentration tested (1.0×10^8 conidia/mL), ensuring robust time-based comparisons.

Mortality data were additionally analyzed using the GENMOD procedure in SAS University Edition (SAS Institute Inc., Cary, NC), with treatment as a fixed effect and replication and trial date as random factors. A generalized linear model (GLM) with binomial distribution and logit link function was applied to assess treatment effects. Means were compared using the Tukey–Kramer adjustment for multiple comparisons at a 5% significance level (Westfall et al., 1999).

We acknowledge that cumulative mortality data may present correlated errors over time. While Probit analysis is widely used for such data, we highlight the importance of more robust time-to-event methods in future work, such as Kaplan–Meier survival curves, which better account for censoring and time dependency (Throne et al., 1995).

3. Results

All isolates caused significantly higher mortality than the control, and fungal infection was confirmed in over 90% of cadavers through sporulation in moist chambers. Statistical analysis revealed significant differences among isolates in terms of mortality rates ($p < 0.05$), as indicated by non-overlapping group letters in Figure 1. Isolates IBCB425, IBCB227, and IBCB220 were selected based on their consistently higher performance compared to others (Tukey–Kramer test, $p < 0.05$). Among the eleven isolates tested, IBCB425 (*Metarhizium anisopliae*), IBCB227 (*Beauveria bassiana*), and IBCB220 (*Cordyceps farinosa*) were selected for further evaluation based on a combination of high cumulative mortality, early onset of visible symptoms, consistent performance across replicates, and confirmed fungal sporulation in over 90% of cadavers

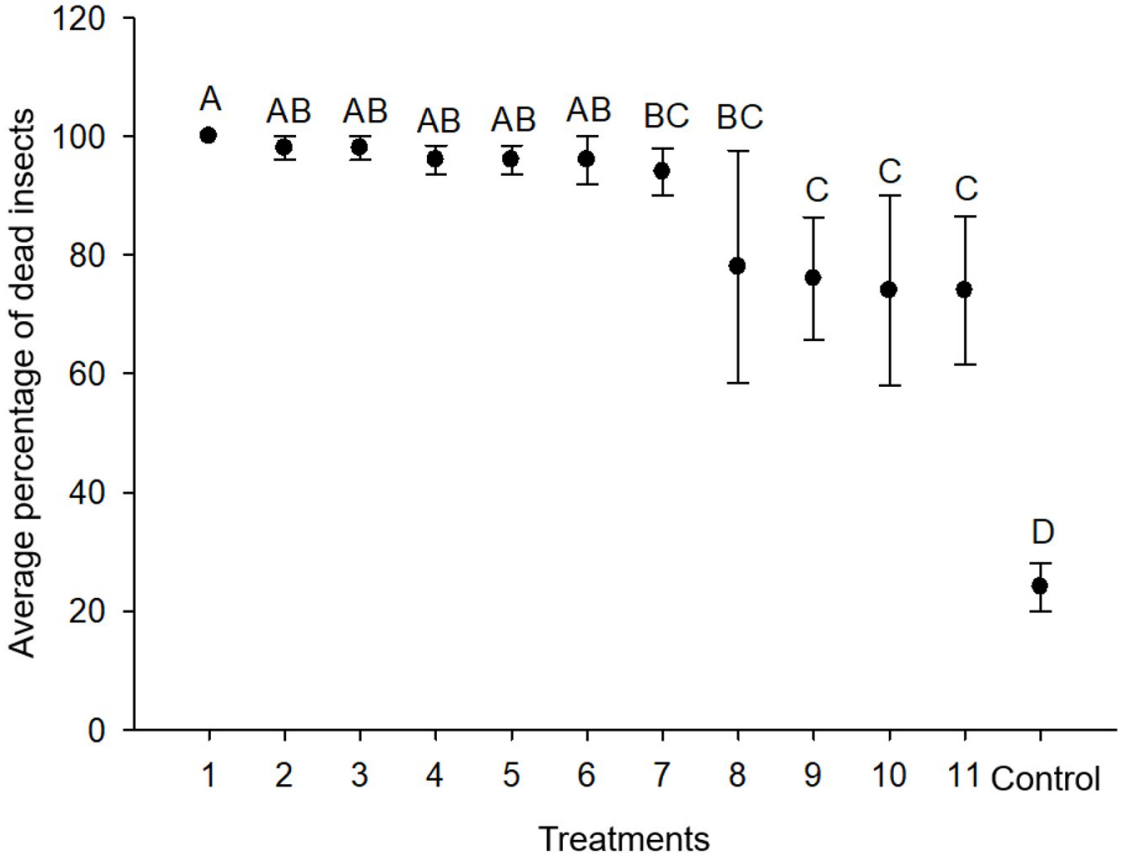

Figure 1. Mortality (%) of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) adults caused by different isolates of entomopathogenic fungi. The isolates tested include *Metarhizium anisopliae* (IBCB425 and IBCB348), *Beauveria bassiana* (IBCB227, IBCB226, IBCB532, IBCB66, IBCB617), *Cordyceps* spp. (IBCB220, IBCB375, IBCB638), and *Sporothrix insectorum* (IBCB79). Control insects were treated with 0.1% Tween 80 only. Mortality was recorded daily and is presented as the cumulative percentage at the end of the bioassay period. Bars represent mean values with standard error (n = 5 replicates; 10 insects per replicate).

(Figure 1). Additionally, IBCB425 and IBCB227 are known for their viability and production potential, making them strong candidates for bioinsecticide development. These criteria ensured the selection of isolates that are not only pathogenic but also practical for future formulation and field use.

In the temperature assay, the three most virulent isolates *B. bassiana* (IBCB227), *M. anisopliae* (IBCB425), and *C. farinosa* (IBCB220) were evaluated at 20, 25, and 30 °C. Mortality rates varied by isolate and temperature (Table 2). According to the Tukey–Kramer test ($p < 0.05$), mortality at 20 °C was significantly lower for *C. farinosa* (IBCB220) compared to its efficacy at 25 and 30 °C. For *M. anisopliae* (IBCB425), mortality remained statistically similar across temperatures ($p > 0.05$), confirming its thermal resilience. Statistically significant differences ($p < 0.05$) between isolates were also observed within each temperature group, as indicated by different capital letters in Table 2. At 25 °C, all isolates caused ≥90% mortality, but at 20 °C, the efficacy of *C. farinosa* (IBCB220) dropped to 66%, while *M. anisopliae* (IBCB425) remained highly effective across all tested temperatures. Fungal infection was confirmed in more than 90% of cadavers through post-mortem sporulation, reinforcing the pathogenic origin of mortality.

Subsequent bioassays determined the lethal concentrations (LC_{50}) and lethal times (LT_{50}) of IBCB227 and IBCB425 for nymphs and adults. The LC_{50} values were 1.10×10^7 conidia/mL for IBCB227 and 2.10×10^6 conidia/mL for IBCB425 (Table 3). LC_{50} values were calculated based on cumulative mortality recorded on the sixth day post-inoculation, which corresponded to the point at which mortality plateaued. LT_{50} values were estimated using mortality data from the highest concentration tested (1.0×10^8 conidia/mL), as this dose yielded the most consistent and complete mortality curves for time-based analysis. The LT_{50} values were 4.43 and 3.75 days for IBCB227 and IBCB425, respectively, indicating slightly faster efficacy for IBCB425. Probit analysis indicated that LC_{50} and LT_{50} values differed significantly between isolates, although the 95% confidence intervals overlapped slightly. IBCB425 exhibited a significantly lower LC_{50} than IBCB227 ($p < 0.05$), confirming its higher virulence at lower doses. LT_{50} values also showed a statistically faster lethal action for IBCB425 compared to IBCB227 ($p < 0.05$), further supporting its superior efficacy.

Table 2. Mortality (%) of adult *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) by the isolates IBCB227 (*B. bassiana*), IBCB425 (*M. anisopliae*), and IBCB220 (*C. farinosa*), and in control under different temperatures.

Isolates	Temperature (°C)		
	20	25	30
IBCB227	94 ± 0.245 Ab	96 ± 0.025 Aa	100 ± 0.000 Aa
IBCB425	98 ± 0.020 Aa	94 ± 0.040 Bb	100 ± 0.000 Aa
IBCB220	66 ± 0.081 Ab	90 ± 0.055 Ca	94 ± 0.040 Ba
Control	12 ± 0.058 Ba	18 ± 0.037 Da	20 ± 0.071 Ca

Table 3. Lethal concentration (LC_{50}) and lethal time (LT_{50}) values for *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) against *T. peregrinus* adults. LC_{50} was estimated based on cumulative mortality at day 6 post-inoculation. LT_{50} was based on data from the highest concentration (1.0×10^8 conidia/mL).

Isolated	LC_{50}[a]	LT_{50} (days)[a]
IBCB227	1.10×10^7 (1.80E+06 - 9.10E+07) A	4.43 (4.02 - 4.91) A
IBCB425	2.10×10^6 (3,40E+05 - 1.60E+07) A	3.75 (3.46 - 4.07) A

[a]Estimated means of LC_{50} followed by the same capital letter per column do not differ by probit analysis. Values are means of two independent trials (n = 50 insects per treatment), with 95% confidence intervals in parentheses.

Table 4. Cumulative mortality of nymphs and adults of *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) with different conidia/mL concentrations (Conc.) of *Beauveria bassiana* (IBCB227) and *Metarhizium anisopliae* (IBCB425) six days after their application.

Conc.	IBCB227		IBCB425	
	Nymph[a]	Adult[a]	Nymph[a]	Adult[a]
10^4	42 ± 9.2Ba α	50 ± 7.1B a α	42 ± 15.0B b α	82 ± 9.7B a α
10^5	80 ± 7.1 A a α	44 ± 10.8B b β	54 ± 11.6B b β	84 ± 4.0A a α
10^6	68 ± 13.9A b α	88 ± 9.7A a α	78 ± 12.0B b α	87 ± 1.2A a α
10^7	84 ± 16.9A a α	96 ± 4.0A a α	92 ± 3.74A a α	96 ± 2.4A a α
10^8	100 ± 0.0A a α	96 ± 4.0A a α	100 ± 0.0A a α	100 ± 0.0A a α

Means followed by the same uppercase letter, per column, lowercase letter per row, or Greek letter per row, do not differ according to the Tukey–Kramer test (Westfall et al., 1999) at a significance level of 5%. Uppercase letters compare concentrations per insect stage and isolate; lowercase letters compare insect stages per concentration and isolate; and Greek letters compare isolates per insect stage and concentration.
[a]Developmental stage of *Thaumastocoris peregrinus*.

Mortality varied between developmental stages and concentrations (Table 4). For example, IBCB227 at 1.0×10^5 conidia/mL caused 80% mortality in nymphs and 44% in adults, whereas IBCB425 at the same concentration resulted in 54% and 84% mortality, respectively. These results highlight a statistically significant dose-dependent response ($p < 0.05$), as well as differences in susceptibility between developmental stages, confirmed by pairwise comparisons. For example, IBCB227 at 1.0×10^5 conidia/mL caused significantly higher mortality in nymphs than in adults (Tukey–Kramer test, $p < 0.05$), whereas the reverse was observed for IBCB425.

Together, these findings support the high virulence and temperature resilience of IBCB425 and IBCB227, confirming their potential as biological control agents for *T. peregrinus*.

4. Discussion

The potential of *M. anisopliae*, *B. bassiana*, *Cordyceps* spp., and *S. insectorum*, with their conidia applied in suspension, to manage *T. peregrinus* was demonstrated. Different isolates of *M. anisopliae*, including IBCB425 and IBCB348, caused mortality of up to 100% to other pests, such as the spittlebug *Mahanarva fimbriolata* Stål (Hemiptera: Cercopidae) (Loureiro et al., 2015), the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae), *Aulacaspis tubercularis* (Hemiptera: Diaspididae) (Nawaz et al., 2022), and *Icerya seychellarum* (Hemiptera: Monophlebidae) (Sayed et al., 2019). *Metarhizium anisopliae* caused 100% mortality in laboratory-reared adults *of Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Orduño-Cruz et al., 2015). The specificity of this fungus for Hemiptera is high but lower than that of *B. bassiana*, as its hosts also

include 204 species of the Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera orders (Veen, 1968). The specificity of isolates to species of certain insect orders varies with characteristics such as the place of origin and virulence genes (Zimmermann, 2007). The pathogenicity of *B. bassiana* and *Cordyceps* spp. isolates to *T. peregrinus* is consistent with reports of mortality ranging from 37% to 80.1% for this insect with *B. bassiana* isolates and 87% with *Cordyceps* sp. at a concentration of 1.0×10^8 conidia/mL (Lorencetti et al., 2018), with the same concentration used in this study. Native entomopathogenic fungi can increase the sustainability of forest pest management, as reported for *B. bassiana*, *Cordyceps* sp., and *Zoophtora radicans* (Wilcken and Oliveira, 2015; Jordan et al., 2021; Domingues et al., 2022a, b). The fungus *S. insectorum* has been used to manage the rubber lace bug *Leptopharsa hevea* Drake & Poor (Hemiptera: Tingidae) (Li et al., 2010), but these are the first reports of its pathogenicity to *T. peregrinus*.

The susceptibility of *T. peregrinus* to entomopathogenic fungi, specifically *M. anisopliae*, confirms reports for this insect (Soliman et al., 2019). The faster mortality of *T. peregrinus* by *M. anisopliae* isolates than by *S. insectorum* is related to the high virulence of the former fungus to Hemiptera, with reports of this isolate being produced and used in the management of other insects and registered against *M. fimbriolata* in Brazil (Loureiro et al., 2005; Lima et al., 2014; AGROFIT, 2018). The potential of the fungus *S. insectorum* against *Leptopharsa heveae* Drake and Poor (Hemiptera: Tingidae) in 1986 in the Amazonas was demonstrated, with 93% mortality of nymphs and 76% mortality of adults of this insect (Celestino Filho and Magalhães, 1986), values similar to those reported for *T. peregrinus*.

Variations in the pathogenicity of *M. anisopliae*, *B. bassiana*, and *C. farinosa* to *T. peregrinus* with temperature, with values greater than 30°C, have also been observed for the first fungus, which is present between 25 and 30°C, with *Maruca vitrata* Fabricius (Lepidoptera: Crambidae) (Tumuhaise et al., 2018). The pathogenicity of *B. bassiana* to *Triatoma infestans* Klug (Hemiptera: Reduviidae) was greater at 34°C (Lecuona et al., 2005), confirming the reduction in germination, growth, viability, and pathogenicity of fungi at inadequate temperatures. High temperatures can inactivate microorganisms before they contact the host or accelerate their growth within the insect. In contrast, low temperatures can reduce or stop the germination and growth of the fungus, hindering or prolonging infection (Zimmermann, 2008). The fungi *M. anisopliae*, *B. bassiana*, and *C. farinosa* are mesophiles, with optimal temperatures of 15 to 35°C, 23 to 28°C, and 20 to 30°C, respectively, but with variations among their isolates (Zimmermann, 2007, 2008; Lecuona et al., 2005). This increases the possibility of using entomopathogenic fungi to manage *T. peregrinus* in different regions of Brazil, most of which have warmer climates, including the states of Espirito Santo, Mato Grosso do Sul, Minas Gerais, Rio Grande do Sul, Rio de Janeiro, São Paulo, Bahia (Lima et al., 2010), Paraná (Barbosa et al., 2010), Santa Catarina (Savaris et al., 2011), Goiás (Pereira et al., 2013), and Pará (Saliba et al., 2019). Differences between isolates facilitate the regionalization of fungal use in biological control in countries such as Brazil, with varying temperatures between its states. Therefore, understanding the phenotypic plasticity of each isolate is important for better use of biological products.

The LC_{50} values of 2.1×10^6 and 1.1×10^7 conidia/mL for the isolates IBCB425 and IBCB227, respectively, for *T. peregrinus* are lower than those of three commercial products based on *M. anisopliae* for this insect, which are 3.2, 3.3, and 3.6×10^7 conidia/mL (Soliman et al., 2019). The LC_{50} of a commercial product based on the isolate IBCB425 for *Glycaspis brimblecombei* Moore (Hemiptera: Psyllidae) was 3.8×10^5 conidia/mL, which was lower than that reported in the present study, but this isolate has not been registered to manage *T. peregrinus* (Dal Pogetto et al., 2011). The LC_{50} values of *M. anisopliae* and *B. bassiana* in the management of *Aphis craccivora* Koch (Hemiptera: Aphididae) were 2.3×10^6 and 1.3×10^8 conidia/mL, respectively (Mweke et al., 2018), with the concentration of the first fungus similar to that used in this study for *T. peregrinus*. However, the LC_{50} values can differ between entomopathogenic fungi and host insects, as reported for *Myzus persicae* Sulzer (Aphidomorpha: Aphididae); *Jacobiasca formosana* Paoli (Hemiptera: Cicadellidae); *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodoidea); and *Stephanitis nashi* Esaki & Takeya (Hemiptera: Tingidae), with LC_{50} values of 6.7×10^4, 1.33×106, 3.6×10^6, and 1.2×10^7, respectively, for the fungus *B. bassiana* (Bb-202) (Bugti et al., 2018).

The LT_{50} values of IBCB425 and IBCB227 were lower than those of commercial products against *G. brimblecombei*, suggesting that the virulence of the fungi *M. anisopliae* and *B. bassiana* may be greater, as 50% of the insects died between 1.4 and 2.3 days (Dal Pogetto et al., 2011). The lower LT_{50} of the isolate IBCB425 for *T. peregrinus* than for *G. brimblecombei* reinforces the report that this isolate is less virulent to *Mahanarva fimbriolata* Stål (Hemiptera: Cercopidae), with a LT_{50} of 4.26 at a concentration of 1.0×10^8 (Freitas et al., 2012). The LT_{50} values of 3.75 and 4.43 days observed for *M. anisopliae* (IBCB425) and *B. bassiana* (IBCB227), respectively, are within the expected range for entomopathogenic fungi acting against hemipterans (Zimmermann, 2007). These values reflect a biologically plausible infection cycle, involving spore adhesion, germination, cuticle penetration, and host colonization. Any previous implication of mortality within 1–2 days has been clarified, and non-standard metrics such as the "mortality speed index" have been removed from the manuscript to avoid misinterpretation.

While LT_{50} values in this study were calculated using Probit analysis based on cumulative mortality data, we recognize that this approach does not fully account for the correlated nature of time-series mortality observations. The statistical approach described by Throne et al. (1995) provides a more appropriate framework for analyzing time-to-event mortality data. The relatively high mortality of *T. peregrinus* nymphs and adults at relatively high concentrations, as well as the mortality of nymphs at the three lower concentrations of IBCB425, may be due to the relatively high susceptibility of adults to fungal infections, highlighting the variation in susceptibility to fungi across insect developmental stages, which is important

for effective fungal application (Sedighi et al., 2013). The LT_{50} values observed for *M. anisopliae* IBCB425 (3.75 days) and *B. bassiana* IBCB227 (4.43 days) are consistent with those reported in studies involving *T. peregrinus* and other hemipteran pests under laboratory conditions. For instance, reported LT_{50} values ranging from 3.8 to 5.2 days for various Metarhizium isolates tested against *T. peregrinus*, reinforcing the virulence potential observed in our trials (Soliman et al. 2019). These values are biologically relevant and reflect realistic infection dynamics, including adhesion, penetration, and internal colonization of the host cuticle. The mortality of *M. fimbriolata* was greater at the highest concentration of *M. anisopliae* (IBCB348), at 1.2×10^7 conidia/mL (Loureiro et al., 2005), and that of *G. brimblecombei* increased with the concentration of conidia of the fungi *B. bassiana*, *M. anisopliae* and *Lecanicillium lecanii* (Dal Pogetto et al., 2011). Successful infection occurs when the fungus completes its cycle and penetrates the insect cuticle, which is composed of polysaccharide polymers incorporated into a protein matrix (Vega et al., 2012). However, the rupture of the insect cuticle during molting can stop the infection if the fungus has not penetrated and reproduced in the host hemocoel. Entomopathogenic fungi have developed mechanisms to adhere to and penetrate the host cuticle, which has evolved to resist pathogens through various means, including immune responses (Ortiz-Urquiza and Keyhani, 2013). Rapid molting reduces the mortality of aphids and *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) caused by entomopathogenic fungi (Vandenberg et al., 1998; Kim and Roberts, 2012), indicating that this process reduces or prevents infection by quickly removing conidia.

The potential of the entomopathogenic fungi *Beauveria bassiana*, *Cordyceps* spp., *Metarhizium anisopliae*, and *Sporothrix insectorum* to manage *T. peregrinus* is high, with a particular emphasis on *M. anisopliae*. The pathogenicity of the isolates *Beauveria bassiana* (IBCB227), *M. anisopliae* (IBCB425), and *Cordyceps farinosa* (IBCB220) to *T. peregrinus* was greater at temperatures of 25 and 30°C, with *B. bassiana* (IBCB227) and *M. anisopliae* (IBCB425) at concentrations of 1.0×10^7 conidia/mL and 2.10×10^6 conidia/mL, respectively, causing 50% mortality in nymphs and adults of *T. peregrinus*. The feasibility of integrated management of *T. peregrinus* in eucalyptus plantations with these isolates of entomopathogenic fungi is high, providing a sustainable biological control approach for insects and reducing their dependence on toxic molecules.

Although our study did not include the isolation of fungi from naturally infected insects, natural occurrences of fungal infections in *T. peregrinus* have been documented in Uruguay (Corallo et al., 2019) and Brazil (Mascarin et al., 2012; Lorencetti et al., 2017). Future studies integrating environmental sampling and native strain characterization will be critical for improving the selection of environmentally competent bioagents.

Our findings align with previous studies reporting effective control of *T. peregrinus* using EPF (Lorencetti et al., 2018; Santos et al., 2018; Velozo et al., 2023; Wilcken et al., 2019) and confirm the importance of selecting isolates with both high virulence and temperature resilience. The superior performance of *M. anisopliae* IBCB425, particularly under both moderate and high temperatures, reinforces its candidacy for biological control programs. Considering the increasing restrictions on chemical control in forest systems, these results contribute to advancing sustainable pest management strategies against this invasive pest. Future field studies will be crucial to validate the performance of these isolates under fluctuating environmental conditions and large-scale application scenarios.

The results demonstrated that *M. anisopliae* IBCB425 and *B. bassiana* IBCB227 are effective entomopathogenic fungi against *Thaumastocoris peregrinus* under different temperature conditions. The consistent virulence of IBCB425 across all tested temperatures and its lower LC_{50} and LT_{50} values highlight its potential as a thermotolerant bioagent. These findings support the selection of fungal isolates not only for pathogenicity but also for performance under ecologically relevant thermal ranges. This approach aligns with the development of robust, sustainable biological control strategies for eucalyptus plantations, where temperature variation is a critical factor. Future studies should focus on field validation and formulation development to advance these candidates into operational pest management tools.

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Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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