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ORIGINAL ARTICLE – Silviculture

Virulence and Selection of *Beauveria Bassiana* for the Control of *Thaumastocoris Peregrinus*

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Abstract

This work aimed to assess the virulence of *Beauveria bassiana* isolates in different concentrations on *Thaumastocoris peregrinus* (Hemiptera, Thaumastocoridae) and select them to control this insect pest. Suspensions of IBCB35, IBCB66, IBCB18, IBCB01 and JAB06 isolates were tested in the concentrations of zero, 1×10^4, 1×10^6, 1×10^8 conidia/ml, applied on the eucalyptus leaves used to feed the insects. Dead insects were disinfested daily and placed in a humid chamber to confirm the death by fungus. Mortality at the end of the experiment ranged from 12% for IBCB66 to 48% for IBCB18 at a concentration of 1×10^4 conidia/ml, between 32% for IBCB66 and 68% for JAB06 at a concentration of 1×10^6 conidia/ml, and between 60% for IBCB01 and 84% for JAB06 at the concentration 1×10^8 conidia/ml. The assessed isolates were efficient in controlling the insect, especially JAB06.

Keywords: silviculture, forest entomology, entomopathogenic fungi.

1. INTRODUCTION AND OBJECTIVES

Thaumastocoris peregrinus Carpinteiro & Dellapé 2006 (Hemiptera, Thaumastocoridae) (common name bronze bug) is a leaf-sucking insect of thin leaves and eucalyptus branches, capable of causing significant damage and making it impossible to maintain and manage new plantations in countries where its occurrence is recorded (Barbosa et al., 2010; Wilcken et al., 2010). Its rapid dissemination capacity and the severity of the injuries caused by the bronze bug in eucalyptus plantations have drawn the forest sector's attention, which aims to develop efficient strategies to control this insect pest.

Among the methods recommended by Integrated Pest Management, control by biological agents is an important tactic against the eucalyptus bronze bug (Barbosa et al., 2010), and the use of entomopathogenic fungi is one of the best strategies. Records show fungi occurrence of the Entomophthorales order, and the *Beauveria bassiana* (Balsamo-Crivelli 1835) Vuillemin 1912 species associated with the bronze bug (Mascarin et al., 2012; Wilcken et al., 2010).

The *B. bassiana* species has been widely studied due to its wide action spectrum and easy laboratory productivity. It is also used as a biological agent to control several insect pests (Alves et al., 1998). Once in contact with the insect, the spores or hyphae of the fungus germinate and secrete enzymes that degrade the host's cuticle, penetrating inside. After penetration, the fungus produces a toxin called Beauvericin, which decreases the insect's immune defenses, causing its death (Azevedo et al., 1999).

Considering the scarce literature involving the relationships between *B. bassiana* and *T. peregrinus* fungi, in addition to the challenge of establishing effective programs in the biological control of this insect, studies conducted investigating the potential use of these agents in controlling the eucalyptus bronze bug are required. This study proposed to investigate the virulence of the *B. bassiana* fungus isolates in different

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concentrations on *T. peregrinus* adult insects under laboratory conditions, and then to select the best strains to control the eucalyptus bronze bug.

2. MATERIALS AND METHODS

The bioassay selection was conducted at the Genetics and Biotechnology Laboratory facilities of the Forest Engineering Department at the Federal University of Jequitinhonha and Mucuri, JK Campus, in Diamantina, Minas Gerais, Brazil.

The *Beauveria bassiana* fungal isolates used were JAB06, IBCB35, IBCB01, IBCB18 and IBCB66, provided by the São Paulo State University, Jaboticabal Campus. For the bioassay, the isolates were put into Petri dishes containing pre-autoclaved BDA culture medium under aseptic conditions. The plates were kept in air-conditioned BOD chambers (26 °C ± 0.5 °C, 12 h photophase and 70% ± 10% relative humidity) for 14 days for the pathogen growth and sporulation.

After the incubation period, hyphae and conidia formed on the surface of the culture medium were collected with the aid of a scalpel and transferred to Erlenmeyer flasks containing 50 ml of potato-dextrose (BD) liquid culture medium in triplicate. After inoculation, the flasks were kept in a BOD type air-conditioned chamber (26 °C ± 0.5 °C, 12 h photophase and 70% ± 10% relative humidity) for 14 days for developing the fungus.

To prepare the inoculum suspensions, the culture medium containing the fungal colonies was filtered and the mycelial mass together with the conidia were suspended in a sterile solution composed of 100 ml of sterile distilled water plus the adhesive spreader (Tween® 80) at 0.05% v/v. After grinding in an electric mixer, the inoculum concentration present in the initial suspension (adjusted to the concentration 1×10^8 conidia/ml) was quantified with the aid of a Neubauer chamber under optical microscopy, according to the quantification method adopted by Alves & Moraes (1998). The other concentrations were then adjusted from the initial solution: 1×10^4 and 1×10^6 conidia/ml, plus the control composed of sterilized distilled water and the adhesive spreader at 0.05% v/v.

Microculture technique and direct examination in microscopy slides determined the viability of the spores to guarantee an experimental standard before applying suspensions to the bugs. The germination percentage was measured 12 h after inoculation, counting 150 conidia per field between germinated and non-germinated.

For the bioassay, healthy leaves of similar coloring and standardized size were collected from an Eucalyptus hybrid, whose identification could not be performed, but which was proven to be susceptible to the bronze bug's attack. The leaves were immersed in the conidial suspensions and held in laminar flow chamber for 30 minutes to eliminate excess moisture. Then, five *T. peregrinus* adults were deposited on the leaves surface, which were placed in Petri dishes previously autoclaved.

The petiole of each leaf was arranged in Eppendorf-type polypropylene tubes containing 1.5 ml of distilled water through a small hole in the tube cap. This procedure enabled maintenance of foliar turgidity and prevented the insects from having contact with water.

After inoculation, the plates were sealed with PVC film and kept in a BOD-type heated chamber at 26 °C ± 0.5 °C, 12 h photophase and 70% ± 10% relative humidity for 10 days. Daily evaluations were performed throughout the experimental period, observing the presence of dead individuals.

To confirm insect mortality by the pathogen, daily bronze bugs in each replicate were disinfested by immersion in 70% ethanol, 2% sodium hypochlorite and distilled water for 2 minutes each, and then transferred to chambers prepared in autoclaved Petri dishes kept in a BOD-type heated chamber at 25 °C ± 0.5 °C and relative humidity of 70% ± 10% in the dark in order to allow the pathogen's sporulation on the insects. The entire procedure was performed aseptically in a laminar flow chamber to avoid contamination by microorganisms. The obtained spores were aseptically transferred to Petri dishes containing sterilized BDA culture medium and grown in an air-conditioned room under optimal conditions to develop the isolates for the fungus recovery and confirmation of its pathogenicity to the insect.

The selection of *B. bassiana* isolates was based on the *T. peregrinus* mortality percentage by the virulence assessment of each isolate at different concentrations.

The bioassay was organized in a completely randomized design in a factorial scheme with two factors + one control, with one factor being the isolate (IBCB35, IBCB66, IBCB18, IBCB01 and JAB06) and the other the concentration (control, 1×10^4, 1×10^6, 1×10^8 conidia/ml). The experimental unit consisted of a Petri dish containing a eucalyptus leaf with five adult *T. peregrinus* insects. Five replicates were established for each treatment.

Results were analyzed considering the different conidial concentrations of assessed isolates and the mortality rates of *T. peregrinus* adults submitted to the treatments. Data were previously normalized and then subjected to analysis of variance (Anova) and the means compared by the Tukey test at 5% significance. The analyses of the obtained data were carried out using the R statistical software program (R Core Team, 2014).

3. RESULTS

The symptoms caused by the bronze bug's sucking habit were perceptible on the second day after transferring the

insects to the eucalyptus leaves, observing the occurrence of chlorosis caused by the insect attack.

The longer assessment period enabled observing the action spectrum of the *B. bassiana* isolates, as well as the behavioral characteristics of the bronze bug, such as egg deposition, development of hatched nymphs and insect-plant relationships during the experimental period.

The low spore viability in the assessed suspensions reached 68.59% for IBCB35, 68.81% for IBCB01, 69.33% for IBCB66, 69.70% for IBCB18 and 69.92%% for JAB06, which ensured the ability of the suspensions to cause infection in the insects and standardization between them. Thus, it was possible to assure the same action conditions for the different isolates used in the tests. Mortality was confirmed by visualizing pronounced bowing of the bronze bugs' legs, which were no longer fixed on the eucalyptus leaves, and also by total lack of mobility.

In relation to the mortality percentage of *T. peregrinus* over time, 100% of dead insects were recorded from the fifth day of assessment for IBCB66 and IBCB18 isolates, when 1×10^8 conidia/ml was considered. The IBCB66 isolate was tested at concentrations of 1×10^4 and 1×10^6 conidia/ml, and only reached this result on the ninth day, while total insect mortality was only observed on the tenth day for the IBCB18 isolate at a concentration of 1×10^6 conidia/ml, when 92% of dead insects were also observed for the concentration of 1×10^4 conidia/ml. The IBCB35 and JAB06 isolates were tested at the highest concentration and reached 100% mortality for *T. peregrinus* on the sixth day of assessment. However, both concentrations of 1×10^4 and 1×10^6 conidia/ml presented total insect mortality on the seventh day. The IBCB01 isolate also only promoted 100% accumulated mortality on the seventh day of assessment for the concentrations of 1×10^8 and 1×10^6 conidia/ml, and at the end of the experimental period, the concentration of 1×10^4 registered 88% of dead insects. Ten days after application of treatments, insects in the control group had accumulated mortality rates between 84% and 92% (Figure 1).

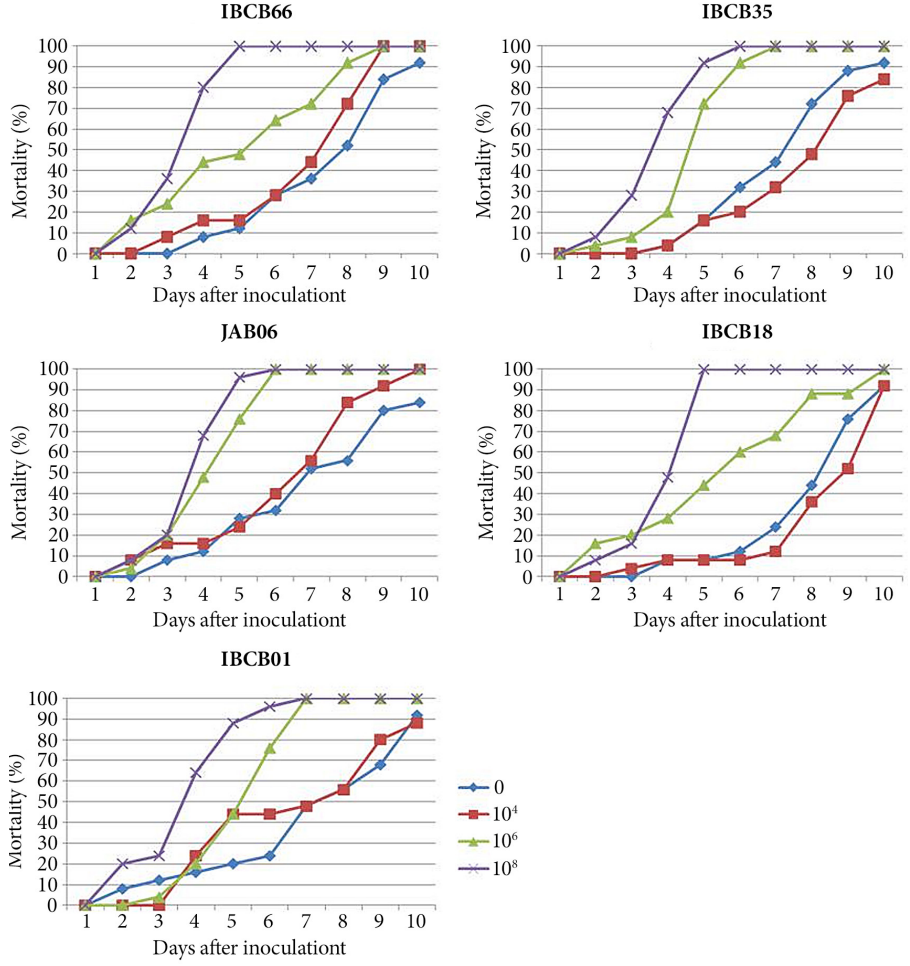

Figure 1. Daily mortality percentage observed in *Thaumastocoris peregrinus* for treatments with the IBCB66, IBCB35, JAB06, IBCB18, and IBCB01 *Beauveria bassiana* isolates at concentrations 1×10^4, 1×10^6, 1×10^8 conidia/ml and the control concentration.

The dead insects representative of the control group of each isolate did not demonstrate conidiogenesis or contamination by other fungi, thus indicating that the superficial disinfestation was in fact efficient. Conversely, five days after being subjected to the humid chamber, sporulation was visible in most of the dead bugs that were exposed to fungal spores. The fungi spores developed on the cadavers and transferred to Petri dishes containing BDA culture medium enabled by multiplying the isolates without having contaminants, confirming their virulence to the insect. The colonies obtained in BDA culture medium showed the mycelium with yellowish-white and cotton-like appearance, with aerial and regular growth. These morphological characteristics are identical to those found in the literature for other *B. bassiana* isolates (Fernandes et al., 2006; Varela & Morales, 1996).

For confirmed mortality, even though the JAB06, IBCB18, IBCB35, IBCB66, IBCB01 isolates tested at the concentration of 1×10^8 conidia/ml, JAB06, IBCB18, IBCB01, and IBCB35 at 1×10^6 conidia/ml, and IBCB18 and JAB06 at 1×10^4 conidia/ml did not present significant differences, the JAB06 isolate (1×10^8 conidia/ml) presented the highest confirmed mortality rate in *T. peregrinus*. This isolate was significantly different from the IBCB01 and IBCB35 isolates tested at the concentration 1×10^4 conidia/ml, and IBCB66 tested at concentrations of 1×10^4 and 1×10^6 conidia/ml, which, in turn, were statistically the same as all isolates tested at the control concentration. The worst confirmed mortality response in *T. peregrinus* was observed for the IBCB66 isolate tested at the concentration 1×10^4 conidia/ml (Table 1).

Table 1. Mortality percentage of the *Thaumastocoris peregrines* adults by the *Beauveria bassiana* fungus isolates tested at different concentrations.

Isolate	Concentration	Mortality (%)	Significance*			
JAB06	10^8	84.00	a			
IBCB18	10^8	68.00	a	b		
IBCB35	10^8	68.00	a	b		
JAB06	10^6	68.00	a	b		
IBCB66	10^8	64.00	a	b		
IBCB18	10^6	64.00	a	b		
IBCB01	10^8	60.00	a	b		
IBCB01	10^6	52.00	a	b	c	
IBCB35	10^6	48.00	a	b	c	
IBCB18	10^4	48.00	a	b	c	
JAB06	10^4	44.00	a	b	c	
IBCB01	10^4	36.00		b	c	d
IBCB66	10^6	32.00		b	c	d
IBCB35	10^4	28.00		b	c	d
IBCB66	10^4	12.00			c	d
IBCB35	0 (control)	0.00				d
IBCB01	0 (control)	0.00				d
IBCB66	0 (control)	0.00				d
JAB06	0 (control)	0.00				d
IBCB18	0 (control)	0.00				d

* Equal letters mean treatments equal to 5% of significance.

4. DISCUSSION

Fungus virulence may vary depending on several factors, including environmental conditions or the presence of elements in the insect cuticle, which may inhibit the action of the pathogen (Boucias & Pendland, 1998). Generally, the rapidity of insect mortality is directly proportional to the conidia concentration (Fargues & Rodrigues-Rueda, 1980) and to the inoculation mode of the pathogen. The IBCB66, IBCB18, IBCB01, IBCB35 and JAB08 *B. bassiana* isolates were pathogenic at all concentrations and the mortality rate increased with increasing concentrations. In general, the JAB06 isolate had higher efficiency in *T. peregrinus.*

The susceptibility of the insect to entomopathogenic fungi may vary depending on their development stage (Alves et al., 1998). In tests with *B. bassiana* on the bronze bug predator *Podisus nigrispinus* Dallas 1851, França et al. (2006) recorded a higher percentage of average mortality for nymphs than for adults.

França et al. (2006) tested the methods of direct spraying of insects and surface treatment of the leaves used in the bioassay, obtaining 59% and 8% of confirmed mortality, respectively. In this work, suspensions of *B. bassiana* isolates were applied indirectly by treating the surface of eucalyptus leaves used as food substrate for the bronze bugs. In treating the leaf surface, the number of conidia that had contact with the insect tends to be less because they are restricted to some regions of the body (Santoro et al., 2007). However, the bronze bug's sucking and behavioral feeding habit on the eucalyptus leaves facilitated the insect infection by the fungus, even if it was only applied on the food substrate.

The application method and the fungal suspension concentrations used enabled virulence expression of the *B. bassiana* isolates on *T. peregrinus*, demonstrating that the application method of the isolates with the surface treatment of the leaves was efficient. In this sense, application by substrate treatment can effectively represent action potential of the fungus.

In field conditions, spraying mycoinsecticides on insect pests can be prevented by the difficulty of direct application to the host, especially in large areas such as those used to plant eucalyptus. In addition, infestation control occurring in the canopy can be difficult and expensive given the height and location of trees in the forest. When sprayed on the plantation, mycoinsecticides can reach a large part of the population of insect pests when they feed on the product deposited on the leaves in the tree canopy. According to Wright (1993), spraying applications of the mycoinsecticides can present similar efficiency to chemical insecticides. Therefore, it is a potential technique to be considered in pest control programs in forest plantations.

Results found in this study corroborate those previously observed by Lorencetti et al. (2011) in adopting a similar methodology when studying the potential of *B. bassiana* for *T. peregrinus* adults control. The obtained mortality percentage results ranged from 46.8% for the IBCB440 isolate to 78.6% for isolate 384, both in concentrations 1×10^8 conidia/ml.

Although they are pathogens of the same species, some *B. bassiana* isolates assessed in this bioassay presented different behavior in relation to the virulence of *T. peregrinus* adults. This frequently observed variation may be related to factors such as susceptibility and/or tolerance of the host insect, as well as characteristics of each isolate, such as virulence, specificity and the genetic variability of each of them (Alves et al., 1988; Vestergaard et al., 1995).

In their studies, Gaitan et al. (2002) observed the occurrence of genetic variation among isolates of native *B. bassiana* populations with the intraspecific distribution obtained from ITS (internal transcribed spacer) and β-tubulin sequence, and also AFLP markers (amplified fragment length polymorphism). Therefore, differences in confirmed mortality percentage of *T. peregrinus* adults found for different isolates can also be justified by the genetic variability among *B. bassiana* isolates.

The *B. bassiana* isolates assessed in this experiment are potentially efficient for *T. peregrinus* control, and the virulence of *B. bassiana* on *T. peregrinus* was generally evident. In addition to reaching 100% total mortality between the fifth and sixth day when tested in the highest concentration, some of the isolates had a high confirmed mortality rate, reaching values above 80%.

Choosing the correct strategy as well as adequate inoculum concentration is of great importance, being a fundamental factor in the successful development and use of entomopathogens as bioinsecticides (Alves et al., 1998). The search for new entomopathogenic fungi isolates is strategically important and may represent an alternative for the biocontrol of the eucalyptus bronze bug in confronting the need to develop efficient methods and low environmental impact for the management of this insect (Barbosa, Belinovski et al., 2012; Barbosa, Santos et al., 2012).

5. CONCLUSIONS

The *B. bassiana* isolates are efficient for *T. peregrinus* control, especially when tested in the highest concentrations, with the best responses being observed for the JAB06 isolate.

The methodology used to assess virulence and selection of *B. bassiana* isolates enables developing other studies that require creating *T. peregrinus* in vitro.

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