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Potential of *Beauveria bassiana* formulations to control *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

Potencial de formulações de *Beauveria bassiana* para controlar *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

Potencial de las formulaciones de *Beauveria bassiana* para controlar *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae)

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Abstract

This study evaluated the potential of four formulations of the entomopathogenic fungus *Beauveria bassiana* for the control of *Thaumastocoris peregrinus*. For this, the leaves of *Eucalyptus dunni* were immersed in each delivery of *B. bassiana*, isolated IBCB 66, and also

in sterile distilled water containing Tween 80® (0.01%) (control), totaling five treatments, with five repetitions each. These leaves were placed in germination boxes containing 10 insects in each and, subsequently, kept in an air-conditioned room (27 ± 2 ° C, 60 ± 10% RH, and 12-h photophase). Record longevity for seven days. The experiment was carried out with adults and also with 3rd instar nymphs of *T. peregrinus*. These formulations were tested for resistance induction, using organic soybean seeds (standard) sown in sand. Ten days after emergence, 40 µL of treatment suspension was competent in 2 mm cuts made on the top of the cotyledon. Phytoalexins and phenylalanine ammonia lyase (PAL) were determined. All formulations caused a reduction in the longevity of *T. peregrinus* when compared to the control, standing out as formulations T1 and T2, both for adults and for 3rd instar nymphs of T. peregrinus. The T2 expansion also promotes an increase in the production of phytoalexins and PAL. Thus, the IBCB 66 isolate from *B. bassiana*, supplied T1, has the potential to control nymphs and adults of *T. peregrinus* and is also a potential resistance inducer.

Keywords: Biological control; Bronze bug; Entomopathogenic fungi; Resistance inducer.

Resumo

Este estudo avaliou o potencial de quatro formulações do fungo entomopatogênico *Beauveria bassiana* para o controle de *Thaumastocoris peregrinus*. Para isto, folhas de *Eucalyptus dunni* foram imersas em cada formulação de *B. bassiana*, isolado IBCB 66, e também em água destilada esterilizada contendo Tween 80® (0,01%) (testemunha), totalizando cinco tratamentos, com cinco repetições cada. Essas folhas foram dispostas em caixas de germinação contendo 10 insetos em cada e, posteriormente, mantidas em sala climatizada (26 ± 2 °C, 60 ± 10% U.R., 12h de fotofase). Avaliou-se a longevidade durante sete dias. O experimento foi realizado com adultos e também com ninfas de 3º ínstar de *T. peregrinus.* Estas mesmas formulações foram testadas na indução de resistência, utilizando sementes de soja orgânica (padrão) semeadas em areia. Dez dias após a emergência, 40 µL da suspensão dos respectivos tratamentos foram aplicados em cortes de 2 mm feitos na parte superior do cotilédone. Fitoalexinas e fenilalanina amônia liase (PAL) foram determinadas. Todas as formulações provocaram redução na longevidade de *T. peregrinus* quando comparadas à testemunha, destacando-se as formulações T1 e T2, tanto para adultos quanto para as ninfas de 3º instar de *T. peregrinus*. A formulação T2 também promoveu aumento na produção de fitoalexinas e PAL. Assim, o isolado IBCB 66 de *B. bassiana*, formulação T1, apresenta potencial para o controle de ninfas e adultos de *T. peregrinus* e também é um potencial indutor de resistência.

Palavras-chave: Controle biológico; Percevejo bronzeado; Fungo entomopatogênico; Indução de resistência.

Resumen

Este estudio evaluó el potencial de cuatro formulaciones del hongo entomopatógeno *Beauveria bassiana* para el control de *Thaumastocoris peregrinus*. Para ello, se sumergieron las hojas de *Eucalyptus dunni* en cada entrega de *B. bassiana*, IBCB 66 aislado, y también en agua destilada estéril conteniendo Tween 80® (0.01%) (control), totalizando cinco tratamientos, con cinco repeticiones cada uno. Estas hojas se colocaron en cajas de germinación que contenían 10 insectos en cada una y, posteriormente, se mantuvieron en una habitación con aire acondicionado (26 ± 2 ° C, 60 ± 10% U.R., fotofase de 12 h). Récord de longevidad durante siete días. El experimento se llevó a cabo con adultos y también con ninfas de tercer estadio de *T. peregrinus*. Estas formulaciones se probaron para la inducción de resistencia, utilizando semillas de soja orgánica (estándar) sembradas en arena. Diez días después de la emergencia, 40 µL de suspensión de tratamiento fueron competentes en cortes de 2 mm hechos en la parte superior del cotiledón. Se determinaron fitoalexinas y fenilalanina amoniaco liasa (PAL). Todas las formulaciones provocaron una reducción en la longevidad de T. peregrinus en comparación con el control, destacándose como las formulaciones T1 y T2, tanto para adultos como para ninfas de 3er estadio de *T. peregrinus*. La expansión T2 también promueve un aumento en la producción de fitoalexinas y PAL. Por tanto, el aislado IBCB 66 de *B. bassiana*, suministrado con T1, tiene el potencial de controlar ninfas y adultos de *T. peregrinus* y también es un inductor potencial de resistencia.

Palabras clave: Control biológico; Chinche del eucalipto; Hongo entomopatógenos; Inductor de resistencia.

1. Introduction

The eucalyptus bronze bug, *Thaumastocoris peregrinus* Carpintero & Dellapé, 2006 (Hemiptera: Thaumastocoridae: Thaumastocorinae), was registered in Brazil for the first time in the state of Rio Grande do Sul, in 2008. The damages caused by this insect are related to its oral sucking lip device, which it uses to suck the sap from the leaves of plants, which start to turn silver, followed by a tan appearance and, consequently, death (Barbosa et al., 2010; Carpinteiro & Dellapé, 2006; Smaniotto et al., 2017; C. Wilcken et al., 2010).

Forest pest control is often carried out using synthetic insecticides, which generates costs, contamination and impacts the environment. Besides, the applicators are also a hindrance to forest certification. To minimize damage to plants and avoid harming the environment, biological control, as an alternative, to synthetic insecticides, and induction of resistance, has been highlighted (Koul & Walia, 2009; Lacey et al., 2015; K. R. F. Schwan-Estrada, 2009; C. F. Wilcken et al., 2019). As regards biological control, entomopathogenic fungi have advantages over other microorganisms, because they can remain in the soil and water as saprophytes and eventually parasite insects, in addition to acting by contact and ingestion (S. B. Alves, 1998)

Beauveria bassiana (Bals.-Criv.) Vuill. is an entomopathogenic fungus that can be found naturally on insects (Prestes et al., 2015) or in the soil (Mascarin & Jaronski, 2016). This fungus has already been registered as causing epizootics in adults of *T. peregrinus* in the field (Lorencetti et al., 2017). Infection of the insects occurs via the integumentary, oral and respiratory systems when the conidia of the fungus adhere to the cuticle of the insect (Ortiz-Urquiza & Keyhani, 2013; Soliman et al., 2019), which is an advantage when compared to other entomopathogens. In addition to causing insect mortality (Lorencetti et al., 2018), this fungus can induce resistance in plants. The reason for this phenomenon is because chitosan (amino polysaccharide, derived from the chitin deacetylation process), a material found in the cell wall of some species of fungi (Azevedo et al., 2007), is capable of inducing disease resistance in plants (Felipini & Di Piero, 2009; Mazaro et al., 2012).

The agricultural and forestry importance of entomopathogenic fungi for the potential control of insects makes them increasingly prominent. In addition, a single species of entomopathogenic fungus has many isolates, each of which may have a unique virulence potential for a particular insect species (Lacey et al., 2015). Add to this the formulation used in commercial products, which may or may not enhance its action. Since *B. bassiana* fungus is produced commercially (Halfeld-Vieira et al., 2016), it is widely used relative to other treatments and in greater culture numbers.

However, each product has a specific formulation, and on coming into contact with plants, in addition to controlling *T. peregrinus*, it can act trigger metabolic routes, in turn, activating the mechanisms of plant resistance. Added to this is the fact that there is no biological product (bioinsecticide) registered for this insect. The objective of this study was to evaluate the potential of four formulation of entomopathogenic *B. bassiana* fungi for the control of *T. peregrinus* and potential to induce plant resistance mechanisms.

2. Methodology

The adult insects of *T. peregrinus* used in the experiments were obtained from the second generation from eggs originating from Embrapa Florestas, which were kept in a mass establishment in the Laboratory of Biological Control I at the Federal University of Technology - Paraná, Campus Dois Vizinhos (UTFPR-DV), Brazil. The rearing was maintained in an air-conditioned room (26 ± 2 °C T, 60 ± 10% relative humidity [RH], 12-h photophase), with no cages, in *Eucalyptus dunnii* (Maiden) branches (Myrtales: Myrtaceae), obtained from an existing settlement in the institution. The branches were collected and disinfected with 1% sodium hypochlorite (NaOCl). Later, these branches were placed in 250-mL Erlenmeyer flasks, with water (changed every 3 days), to maintain their turgidity. For the oviposition of *T. peregrinus*, paper towels were provided, in which the adults deposit their eggs, according to the methodology already described by Barbosa et al., (2016).

2.1 Virulence of *Beauveria bassiana* to *Thaumastocoris peregrinus*

To perform the bioassays, leaves of *E. dunni* were used, without phytosanitary treatment. Before the bioassays, the leaves were disinfected with 1% NaOCl, dried and immersed for 5 seconds in the solution of the respective treatments/formulations: T1, T2, T3 and T4 (four distinct formulations of the fungus *B. bassiana* strain IBCB 66 and 0.01% Tween 80® (0.01%), with T1 and T2 liquid formulations, and T3 and T4 powder) at 1.0×10^{8} conidia mL^{-1} (standard concentration in insect mortality experiments) (Domingues et al., 2020; Lorencetti et al., 2018; Santos et al., 2018). The control (T5) was composed of sterile distilled water containing 0.01% Tween 80. The petiole of each treated leaf was coupled to the lid of a glass (5-mL capacity) filled with water so that the leaf turgidity was maintained. The leaves coupled to the glass were placed inside sterile boxes of transparent polystyrene, gerbox-type (11 cm × 11 cm × 3.5 cm), which were replaced every 3 days, and received no further treatment (Soliman et al., 2019; C. F. Wilcken et al., 2019).

Each treatment consisted of five boxes containing one leaf of *E. dunni*, with 10 insects each, where each box represented a repetition. Two bioassays were conducted; one with adult insects and the other with the 3rd instar nymphs of *T. peregrinus*, of controlled instar and origin. We used 3rd instar because it is at this stage that this insect begins to cause damage to the the plant, in addition, the anterior instars have greater sensitivity to products and handling. The gearboxes, containing the treatments and the insects, were sealed with polyvinyl chloride

film, duly identified and later transferred to an air-conditioned room (26 ± 2 °C, 60 ± 10% RH, and 12-h photophase), considering that these are optimal conditions for the development of fungi and favorable to insects. The mortality and longevity of the insects were evaluated every 12 h for 168 h.

2.2 Potential of *Beauveria bassiana* as a plant resistance inductor

Soybean is a standard plant to evaluate mechanisms of induction of resistance (Schwan-Estrada et al., 2000). Therefore, soybean seeds (*Glycine max* (L.) Merrill), cultivar BRS 284, organic (Gebana® Brasil, Capanema, Brazil) were used to assess the potential of *B. bassiana* as a plant resistance inductor. The treatments used were four different formulations of a single *B. bassiana* strain (IBCB 66), and the control consisted of sterilized distilled water. Soybean seeds, pre-sterilized with 1% NaOCl, were seeded in sterilized sand contained in white trays (60 × 40 cm) and kept under greenhouse conditions. No chemical treatments were performed on the seeds, in order to avoid interference in the resistance induction results (Bruzamarello et al., 2018; Locateli et al., 2019; Lorencetti et al., 2015). At 10 days after emergence, the cotyledons were harvested and subjected to a sterilized distilled water washing process.

In the upper part of the cotyledons, a 2-mm cut was performed, which received 40 µL of the suspension of the respective treatments. Each treatment was composed of four replicates, and each replicate contained four cotyledons of soybean, totaling 80 cotyledons. Afterward, the cotyledons were weighed and inserted (n = 4 per plate) into Petri dishes (60 cm × 15 cm), lined with sterilized and moistened filter paper. The plates were then capped and incubated at 27 ± 2 °C T, 60 ± 10% relative humidity [RH], in the dark for 20 h. The cotyledons were removed, placed in glass beakers containing 10 mL of distilled water and then agitated on an orbital shaker at room temperature for 1 h, to extract the glycerol. The solution was filtered through Whatman 41 filter paper, and the absorbance was determined in a spectrophotometer at 285 nm wavelength, as described by Hahn & Albersheim (1978).

2.4 Statistical analysis

The *T. peregrinus* confirmed mortality was evaluated by survival analysis was performed using Kaplan-Meier. The treatments were compared using the log-rank test and the complete analysis was performed using the survival package of the R software. The

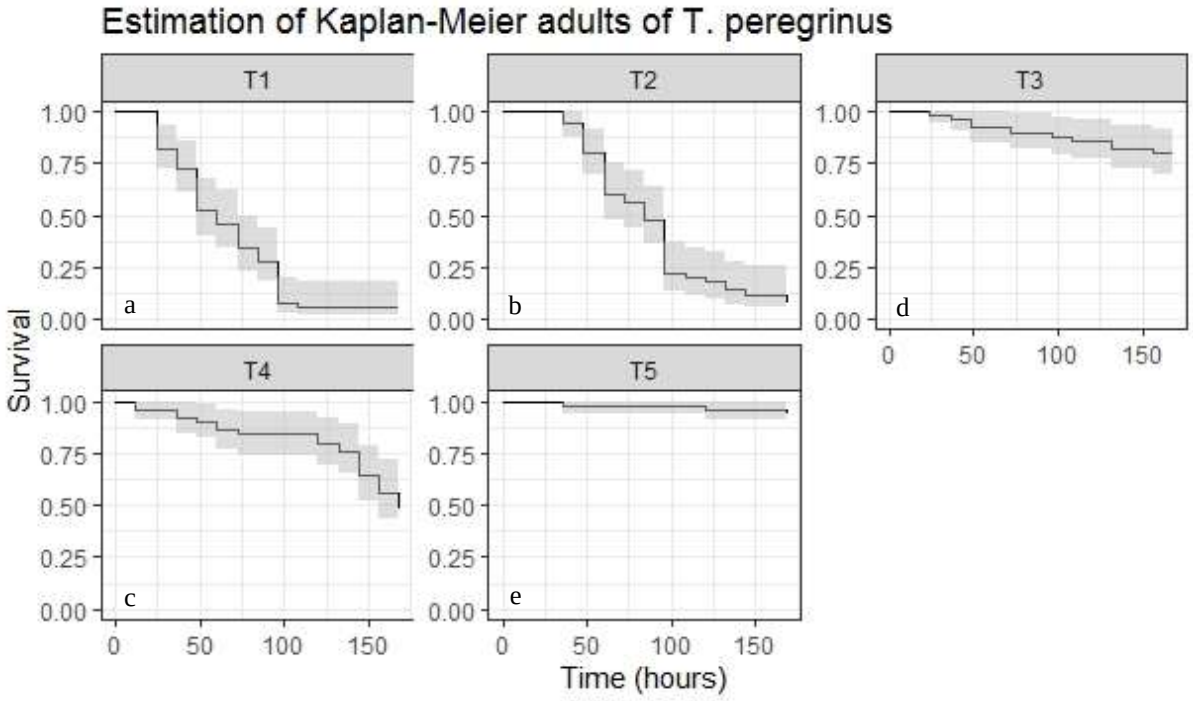
phytoalexins and phenylalanine ammonia lyase (PAL) data of soybean cotyledons were tested for normality, using the Lilliefors test and the Shapiro–Wilk test. As this assumption was satisfied, differences among means were examined by analysis of variance (ANOVA), followed by Scott–Knott's test.

3. Results and discussion

3.1 Virulence of *Beauveria bassiana* to *Thaumastocoris peregrinus*

All the *B. bassiana* strain IBCB 66 tested in this study, caused the mortality of adults from *T. peregrinus*, observed that there was a difference between all treatments, with the highest mortality in formulation T1, followed by T2, T4 and T3 (Figure 1).

Figure 1 - Graph of survival of adults of *T. peregrinus*, by Kaplan-Meier, adjusted to the period (hours) after exposition into four distinct formulations of the fungus *B. bassiana* strain IBCB 66 (T1, T2, T3, T4) at 1.0×10^{8} conidia mL^{-1} or sterile distilled water containing 0.01% Tween 80® (T5). Equal letters indicate that there was no significant difference ($p < 0.05$).



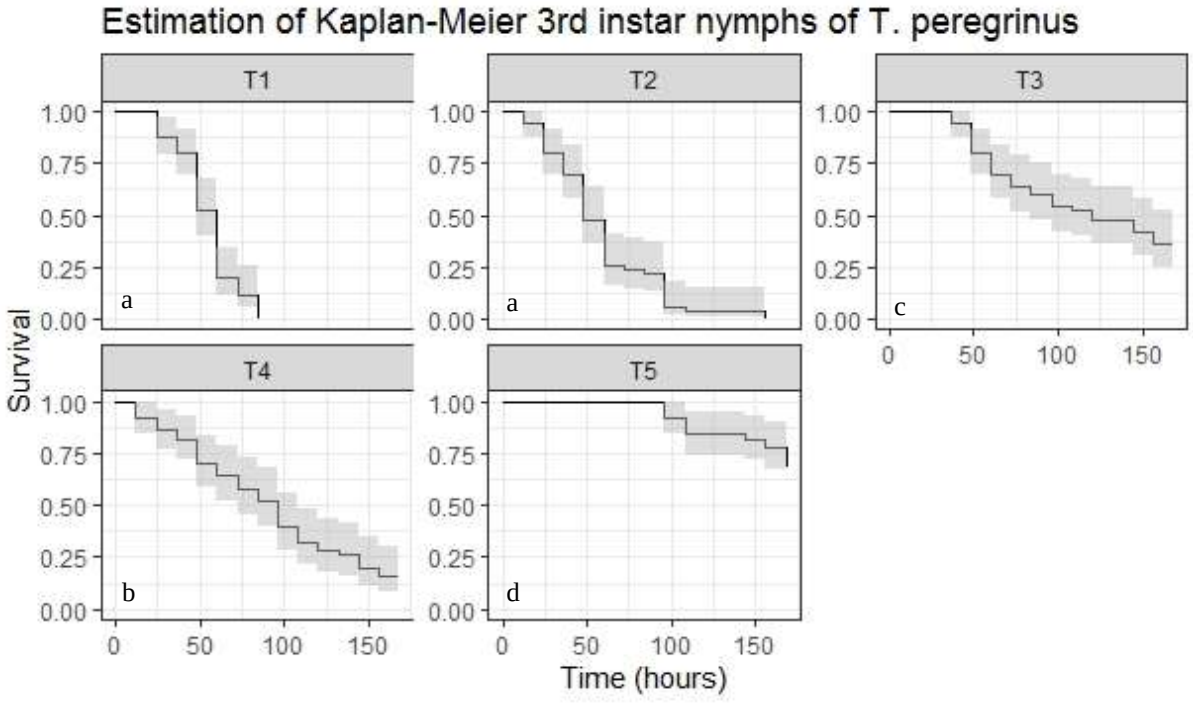
Source: Authors (2020).

When evaluated its action on nymphs, the T1 and T2 formulations, caused higher mortality in nymphs of 3rd of *T. peregrinus* in a shorter period, differing from the other

treatments. The T4 formulation caused lower mortality than that observed when *T. peregrinus* came into contact with T1 and T2 (Figure 2). While the T3 formulation caused lower mortality than other treatments containing the fungus, however, it also differed significantly from the control.

Assessing adult longevity, it is observed that in 100 h approximately 90% of insects had not survived on T1. While in control, mortality was approximately 2% in the same period. The mortality of nymphs in 75 h was higher than 75% in formulations T1 and T2 and in the control treatment all were alive.

Figure 2 - Graph of survival of 3rd instar nymphs of *T. peregrinus*, by Kaplan-Meier, adjusted to the period (hours) after exposition into four distinct formulations of the fungus *B. bassiana* strain IBCB 66 (T1, T2, T3, T4) at 1.0×10^8 conidia mL^{-1} or sterile distilled water containing 0.01% Tween 80® (T5). Equal letters indicate that there was no significant difference ($p < 0.05$).



Source: Authors (2020).

In the present study, four different formulations of *B. bassiana* were tested on nymphs and adults of *T. peregrinus*, among which, two formulations were highlighted. In other studies, isolates of *B. bassiana* and *Isaria* sp. have also been reported to be pathogenic to *T. peregrinus* under laboratory conditions similar and using the same concentration similar to the present research (Lorencetti et al., 2018; Santos et al., 2018; Soliman et al., 2019). Almeida et

al., (2020) studying the virulence of different isolates of *B. bassiana* of *T. peregrinus* found that the accumulated mortality of 60% for IBCB01, 64% for IBCB66, 68% for IBCB35 and IBCB18 and 84% for JAB06 in adults, after 10 days, using the same concentration tested in this study and under laboratory conditions similar. Thus proving, the efficiency of these entomopathogenic fungi in the control of this forest pest.

Various isolates of entomopathogenic fungi have already been studied and tested on other insect pests of the order Hemiptera. Among these, *B. bassiana* isolates show insecticidal potential against nymphs and adults of *Gyropsylla spegazziniana* (Lizer & Trelles) (Hemiptera: Psyllidae) (Alves et al., 2013) *Corythucha arcuata* (Say) (Hemiptera: Tingidae) (Sönmez et al., 2016), *Nezara viridula* Linnaeus (Hemiptera: Pentatomidae) (Raafat et al., 2015) and *Glycaspis brimblecombei* (Dal Pogetto et al., 2011; Dal Pogetto et al., 2011). Likewise, in this study, *T. peregrinus* insects are susceptible to the tested *B. bassiana* formulations.

3.2 Potential of *Beauveria bassiana* as a plant resistance inductor

The use of soybeen cotyledons stands out in research related to the action of eliciting molecules of both biotic and abiotic origin. Thus, soy is considered a model plant in studies related to resistance induction, due to the presence of phytoalexin gliceolin that interact with pathogens that cause stress in plants (Schwan-Estrada et al., 2000). These phytoalexins are secondary metabolites, produced by the plant in response to physical, chemical or biological stresses.

Thus, the formulation of *B. bassiana* T2 was the major inducer of phytoalexin productions in soybean cotyledons, differing from the others (T2, T3 and T4) and the control. For PAL, the T2 treatment also differed from the other treatments and the control, presenting a value of 0.0674 Unit/minute/mg.protein. It should be noted that the highest values for phytoalexins and PAL were obtained in soybean cotyledons submitted to the T2 treatment (Table 1).

Table 1 - Phytoalexins (± SE) and phenylalanine ammonia lyase (PAL) (± SE), in soybean cotyledons exposed to five treatments (T1, T2, T3, T4 and T5). T1, T2, T4 are different formulations of the entomopathogenic fungus *Beauveria bassiana*, and T5, the control, is composed of sterilized distilled water.

Treatment	Phytoalexins Abdorbance (285nm)/fresh.weight.g	PAL Unit/ minute/ mg. protein
B. bassiana (T1)	0.1406 ± 0.012 b	0.0495 ± 0.019 b
B. bassiana (T2)	0.3316 ± 0.059 a	0.0674 ± 0.002 a
B. bassiana (T3)	0.0281 ± 0.007 c	0.0294 ± 0.002 c
B. bassiana (T4)	0.0224 ± 0.005 c	0.0316 ± 0.001 c
Control (T5)	0.0278 ± 0.002 c	0.0363 ± 0.001 c
F	24.20	
p	<0.001	

F: Result in ANOVA, p: percentage of significance. Means followed by the same letter in the column do not differ from one another by the Scott–Knott test ($p < 0.001$). Source: Authors (2020).

In sustainable agriculture, the reduction of the use of synthetic chemicals is of great importance. At the same time, strategies that seek to reduce the commercial use of these products have been gaining momentum, including methods to induce resistance in plants, diseases, and insects. Chitosan is one of the main inducers of plant resistance, as it increases plant production and may also function to control some phytopathogenic microorganisms (Felipini & Di Piero, 2009; Mazaro et al., 2012).

Chitosan acts by binding to receptors present on the cell membrane of plants, imitating the recognition that occurs in the mutually incompatible interaction between the plant and the pathogen. In this way, chitosan can inhibit the proteinases and promote lignification, alter the metabolism of phytoalexins, induce the formation of phenolic compounds, peroxidase and PAL, and activate enzymes, such as chitinases and β-1,3-glucanases (Bautista-Baños et al., 2006; Mazaro et al., 2008; Terry, L Joyce, 2004). Chitosan, obtained from the deacetylation of chitin, is found in the cell wall of some species of fungi, insects and crustaceans (Azevedo et al., 2007). Due to the presence of chitosan in the fungal cell wall, it can be inferred that *B. bassiana* fungus has acted as a resistance inducer in soybean cotyledons in the T2 treatment. The powder formulations of the fungus (T3 and T4) may present some compound that inhibited the activation of the metabolic routes, although other biochemical studies are necessary to prove this observation.

PAL is much studied in plants because it is an enzyme of secondary metabolism (Stangarlin et al., 2011). This enzyme allows the synthesis of compounds, like lignin, flavonoids (anthocyanins), phytoalexins and salicylic acid (Emiliani et al., 2009). Thus, as in

this work, the control presented a small production of phytoalexins, probably induced by the mechanical damage done on the cotyledons. Such behavior could be expected because phytoalexins are secondary metabolites produced by the plant in response to physical, chemical and biological stress (Cavalcanti et al., 2005).

In the quest to obtain healthier fruits without the use of chemical products, work done with chitosan showed that it acts on the systemic resistance of strawberries. Thus, products containing chitosan in its composition (Mazaro et al., 2012), or even entomopathogenic fungi, as in the case of this study, can activate the defense mechanisms in plants.

A study investigating the alcoholic extract of *Eugenia uniflora* (family Myrtaceae) as an inducer of resistance, used 1% chitosan as an inductive reference (Mazaro et al., 2008). Other study exploring alternative phytosanitary products as resistance inductors also used 1% chitosan as the reference resistance inducer (Lorencetti et al., 2015).

The use of bio-insecticides has been prominent in recent years, due to contamination caused by synthetic pesticides. Among the potential biocontrol treatments, the entomopathogenic fungi are being examined on a variety of insect species as a strategy for controlling insect pest, such as the bronze bug *T. peregrinus* on eucalyptus and, a possible use as a resistance inducer.

4. Final Considerations

The four formulations of *B. bassiana* reduced the longevity of *T. peregrinus* adults and 3rd instar nymphs, emphasizing the T1 and T2 formulation in reducing the longevity of nymphs and the formulation T1 for adults. In addition, the formulations of *B. bassiana* also act as resistance inducers, highlighting the T2 formulation that resulted in considerable production of phytoalexins and PAL activity. Thus, all formulations of *B. bassiana* strain IBCB 66 have potential for *T. peregrinus* control. New tests are recommended whenever new formulations are produced or new isolates released on the market.

Studies using *B. bassiana* on *T. peregrinus* should be carried out in the field to better determine the virulence of this biological agent on this insect in eucalyptus plantations. In addition, once the resistance induction in soy has been verified, the tests must still be carried out on eucalyptus in a greenhouse and later in the field.

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