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Susceptibility of adults of the cerambycid beetle *Hedypathes betulinus* to the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Purpureocillium lilacinum*

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Abstract

The cerambycid beetle *Hedypathes betulinus* (Klug) (Coleoptera: Cerambycidae) causes severe damage to yerba mate plants (*Ilex paraguariensis* (St. Hilaire) (Aquifoliales: Aquifoliaceae)), which results in large losses of production. In this study, the pathogenicity of entomopathogenic fungi of the species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae), *Metarhizium anisopliae sensu lato* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae), and *Purpureocillium lilacinum* (Thom) Luangsa-ard, Hywel-Jones, Houbraken and Samson (Hypocreales: Ophiocordycipitaceae) on yerba mate were evaluated. Fifteen isolates of *B. bassiana*, two of *M. anisopliae*, and seven of *P. lilacinum* on *H. betulinus* adults were analyzed under laboratory conditions. The raw mortality rate caused by *B. bassiana* isolates varied from 51.1 to 86.3%, and their LT_{50} values varied between 8.7 and 13.6 d. The isolates of *M. anisopliae* caused 69.6–81.8% mortality, and their LT_{50} values varied between 7.4 and 7.9 d. In contrast, isolates of *P. lilacinum* were not pathogenic. *M. anisopliae* and *B. bassiana* isolates were pathogenic against *H. betulinus* adults, suggesting that they may be useful in biological control programs for insect pests of yerba mate.

Keywords: biological control, pathogenicity, insect pests, yerba mate
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Introduction

Yerba mate plants, *Ilex paraguariensis* (Saint Hilaire) (Aquifoliales: Aquifoliaceae), plantations are attacked by many pests, including the cerambycid beetle *Hedypathes betulinus* (Klug) (Coleoptera: Cerambycidae), which causes severe damage resulting in economical loss in this crop (Casanello 1993). The most severe damage is caused by larval galleries built up or down in the branches and trunks of yerba mate that prevent the normal flow of sap and cause the death of the plant (Alencar 1960). Insecticides, for the control of immature forms and adults, are not recommended because of the risk of toxic residues in the final product (Borges 2007).

In agricultural fields, the entomopathogenic fungal species have been investigated for their potential as the biological control agents because of their role as natural enemies for insects. Conidia that adhere to the surface of the host release extracellular enzymes, including lipases, proteases, and chitinase that help breach the host's chitinous exoskeleton (Pendland et al. 1993, Freimoser et al. 2003, Tscharntke et al. 2005). These fungi have been documented to occur naturally in more than 750 species of hosts and have been used in the development of microbial insecticides (Hajek and St. Leger 1994, Inglis et al. 2001, Shah and Pell 2003).

Until now, most of the studies about these fungi have been based on isolation from cadavers of insects or soil (Vu et al. 2007, Abdo et al. 2008, Glare et al. 2008; Santoro et al. 2008, Brownbridge et al. 2010). In this context, the genera *Paecilomyces*, *Lecanicillium*, *Aschersonia*, *Beauveria*, and *Metarhizium* have been used successfully on experimental or field applications (Monzón 2001, Dos Santos and Pozo 2003, Pucheta et al. 2006). In general, fungi are an excellent alternative to conventional pesticides because they can infect different stages of its hosts' development; they also are nearly pathogenic or not at all to beneficial organisms and humans (Ferron 1977).

Globally, the two most common and studied fungi are the entomopathogenic *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae sensu lato* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) because they are efficient and easily propagated (Rodriguez et al. 2006). *B. bassiana* is a popular registered mycoinsecticide that has a target list of 700 host insect species (Li 1988, Glare and Milner 1991, Humber 1991, Goettel et al. 2000). It is ubiquitous in distribution and is pathogenic to a wide spectrum of arthropods; its host range spans most orders of class Insecta (Butt and Goettel 2000, Lacey et al. 2001, Zimmerman 2007). *M. anisopliae* has a wide host range; it has been documented that they can parasitize more than 300 species of insects of various orders (Gómez et al. 1997).

Purpureocillium lilacinum (Thom) Luangsaard, Hywel-Jones, Houbraken and Samson (Hypocreales: Ophiocordycipitaceae) is a soil fungus with a good potential for biological control. This species has been described as being as efficient as the commonly used nematicides (Dube and Smart 1987, Schenck 2004, Mendoza et al. 2007, Núñez et al. 2012); it is also a controller of insects (Posada et al. 1998, Suh et al. 2002, Gökçe and Er 2005, Wakil et al. 2012) and others arthropods (Fiedler and Sosnowska 2007, Shin et al. 2011, Angelo et al. 2012). According to Bellows (2001), Headrik and Goden (2001), Lanza et al. (2004), and other authors, the use

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of entomopathogenic fungi is an excellent method for the biological control of insects.

The natural occurrence of the fungus *B. bassiana* infecting insects and mites in cultivation of yerba mate (Ribeiro et al. 1994, Dalla Santa et al. 2009) opens different options for its use to improve phytosanitary plant conditions and the quality and productivity of yerba mate, thereby responding to the current demands of the market (Borges et al. 2003). Leite et al. (2000) isolated a strain of *B. bassiana* CG 716 of adult *H. betulinus* collected in field in Ivaí, PR, Brazil, which has been deposited in the Collection of Embrapa Genetic Resources and Biotechnology. This isolation CG 716 was evaluated under field conditions, demonstrating its potential for control of *H. betulinus*. The importance of selection studies are continually made to be the basis for the success of biological control programs using entomopathogenic fungi (Alves 1998, Borges 2007). In Argentina, there are no screening studies of native fungal isolates for controlling insect pests of yerba mate. We believe that this is the first research reported about selection of isolates of entomopathogenic fungi for control of *H. betulinus*.

The objective of this study was to determine the pathogenicity of *B. bassiana*, *M. anisopliae*, and *P. lilacinum* isolates on adults of *H. betulinus* under laboratory conditions.

Materials and Methods

Insects

Hedypathes betulinus adults were collected manually and individually from a yerba mate plantation in Ivaí, Paraná, Brazil. The insects were individualized in plastic containers of 11 × 8 cm, with a perforated lid, and transported to the laboratory. The insects were placed in wooden cages of 60 × 40 × 40 cm and were fed on branches of yerba mate. The cages were kept in controlled room (26 ± 1°C, photoperiod of 14:10 L:D, and 70% RH).

Fungal isolates

From 24 monosporic isolates, 15 corresponded to the fungus *B. bassiana*, two isolates belonged to *M. anisopliae sensu lato*, and seven to *P. lilacinum*. They were obtained from soil samples collected from different crops of yerba mate plantation in the different locations of Misiones province, Argentina (Table 1). Fungal species were identified according to taxonomic keys (Samson 1974; Samson et al. 1988; Humber 1996, Humber 1997; Tzean et al. 1997; Hodge 2006). The fungal isolates were cultured on Sabouraud dextrose agar complemented with 1% yeast extract (SDAY) in 90 mm Petri dishes and incubated for 10 d at 26 ± 1°C and 14 h photophase. After this period, conidia were collected and stored in glass tubes at –10°C. Fungal isolates were deposited in the Mycological Collections of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, La Plata, Buenos Aires, Argentina).

Conidial viability of each isolate was assessed after 24 h using the techniques described by Lane et al. (1988). Conidia were examined under a microscope at 400× magnification. Conidia were considered germinated when germ tubes were longer than conidial length. A total of 600 conidia were evaluated, and relative percent germination was calculated.

Bioassays against *Hedypathes betulinus*

A suspension of conidia in 0.01% Tween 80 from fungal sporulated cultures was prepared, and the concentration was adjusted to 1×10^8 conidia/mL, based on hemocytometer count. For each isolate, the inoculation was performed by immersing 30 adults insects

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Table 1. Details about the entomopathogenic fungi collected in the province of Misiones (Argentina) and used in pathogenicity tests against adult *Hedypathes betulinus*

Fungus species	Accession number	Location	Coordinates	Collection date
B. bassiana	CEP 332	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 333	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 334	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/12/2008
B. bassiana	CEP 335	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/12/2008
B. bassiana	CEP 336	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 337	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
B. bassiana	CEP 338	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
B. bassiana	CEP 339	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
B. bassiana	CEP 340	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 341	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
B. bassiana	CEP 342	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
B. bassiana	CEP 343	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/12/2008
B. bassiana	CEP 344	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 345	Santa Inés	27°31′32.13″S/55°51′47.70″W	01/11/2008
B. bassiana	CEP 347	Santa Inés	27°31′32.13″S/55°51′47.70″W	05/01/2009
M. anisopliae	CEP 349	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
M. anisopliae	CEP 350	Campo Ramón	27°27′18.27″S/55°1′27.02″W	14/05/2009
P. lilacinum	CEP 352	Apóstoles	27°55′10.19″S/55°44′11.01″W	25/06/2009
P. lilacinum	CEP 353	Apóstoles	27°54′24.49″S/55°46′28.76″W	30/06/2009
P. lilacinum	CEP 354	Apóstoles	27°55′10.19″S/55°44′11.01″W	25/06/2009
P. lilacinum	CEP 355	Apóstoles	27°54′24.49″S/55°46′28.76″W	30/06/2009
P. lilacinum	CEP 356	Santa Inés	27°31′32.13″S/55°51′47.70″W	17/02/2009
P. lilacinum	CEP 359	Jardín América	27°3′8.06″S/55°45′18.83″W	04/07/2009
P. lilacinum	CEP 360	Ruiz de Montoya	26°57′54.78″S/55°5′24.36″W	09/03/2010

individually in the conidial suspension for 10 s, then transfering the treated insects to 90 mm diameter Petri dishes lined with filter paper to remove excess suspension of conidia. They were then transferred to plastic cups containing a branch of yerba mate. The cups were closed with a perforated plastic lid. Control insects consisted of adults immersed in sterile distilled water with 0.01% Tween 80. Insects were maintained under controlled conditions as described above. The bioassays were repeated three times. The adults were examined for mortality every 24 h for 15 d. Dead insects were removed and sterilized superficially in 70% alcohol and two successive baths with distilled water. Then they were placed in moist chambers and maintained under the same controlled conditions for the emergence of the mycelium of the fungus. Mortality was confirmed by observing the insects under the stereomicroscope.

Statistical analysis

In each treatment, the cumulative mortality was corrected for control mortality according to Abbott's formula (Abbott 1925). Percentage of germinated conidia and percentage of mortality was analyzed by Statgraphics Centurion 15.2 program (StatPoint 2007). An arcsine transformation was performed to stabilize the variance of germination and percent mortality. A test of homogeneity of variance was performed to detect variation between each experiment. Then, data were submitted to analysis of variance (ANOVA) and Tukey's multiple range tests ($P < 0.05$). Lethal time 50% (LT_{50}) values were calculated by using the statistical software for correlated data developed by Throne et al. (1995).

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Table 2: Cumulative mortality and median lethal time (LT_{50}) of entomopathogenic fungi on adult *Hedypathes betulinus*

Fungus species	Number isolate	Conidial viability (% ± SE)[1]	Mortality (% ± SE)[2]	LT_{50} (days; 95% CI)[3]
Control	-	-	3.3 ± 4.7a	-
B. bassiana	CEP 332	99.6 ± 0.3	61.8 ± 6.0b	11.8 (8.6–16.9)
B. bassiana	CEP 333	91.9 ± 3.2	64.8 ± 14.9b	13.0 (7.8–40.3)
B. bassiana	CEP 334	89.5 ± 7.5	86.3 ± 4.5b	11.6 (10.2–13.4)
B. bassiana	CEP 335	98.1 ± 0.5	85.5 ± 13.9b	10.4 (9.3–11.6)
B. bassiana	CEP 336	98.4 ± 1.2	78.5 ± 18.1b	10.1 (8.5–12.2)
B. bassiana	CEP 337	99.4 ± 0.7	64.8 ± 14.9b	12.1 (9.6–16.0)
B. bassiana	CEP 338	99.8 ± 0.1	82.9± 4.2b	9.6 (7.8N-12.1)
B. bassiana	CEP 339	98.6 ± 0.5	83.3 ± 12.5b	9.7 (7.8–12.1)
B. bassiana	CEP 340	95.8 ± 2.9	68.5 ± 10.0b	8.7 (6.5–11.9)
B. bassiana	CEP 341	100 ± 0.0	65.2 ± 10.6b	9.6 (8.0–11.3)
B. bassiana	CEP 342	99.3 ± 0.7	68.1 ± 18.6b	10.3 (7.9–14.0)
B. bassiana	CEP 343	97.8 ± 0.1	72.6 ± 3.7b	11.9 (10.5–13.7)
B. bassiana	CEP 344	100 ± 0.0	68.5 ± 10.0b	11.9 (10.3–14.1)
B. bassiana	CEP 345	100 ± 0.0	68.9 ± 1.6b	13.6 (12.0–15.9)
B. bassiana	CEP 347	100 ± 0.0	51.1 ± 14.9b	12.2 (10.3–14.7)
Control	-	-	3.3 ± 4.7a	-
M. anisopliae	CEP 349	97.8 ± 0.5	69.6 ± 15.9b	7.4 (3.7–16.0)
M. anisopliae	CEP 350	100 ± 0.0	81.8 ± 19.0b	7.9 (6.8–9.3)
Control	-	-	11.7 ± 8.5a	-
P. lilacinum	CEP 352	97.3 ± 0.7	0.0 ± 0.0b	ND
P. lilacinum	CEP 353	100 ± 0.0	0.0 ± 0.0b	ND
P. lilacinum	CEP 354	100 ± 0.0	0.0 ± 0.0b	ND
P. lilacinum	CEP 355	100 ± 0.0	0.0 ± 0.0b	ND
P. lilacinum	CEP 356	98.4 ± 0.9	0.0 ± 0.0b	ND
P. lilacinum	CEP 359	100 ± 0.0	0.0 ± 0.0b	ND
P. lilacinum	CEP 360	100 ± 0.0	0.0 ± 0.0b	ND

[1]Mean percentage of conidial viability (standard error) obtained after incubation at 25°C during 24 h.
[2]Corrected mortality using Abbott's formula. Within column, means followed by different letters are highly and significantly different ($P \leq 0.01$; Tukey's test).
[3]95% confidence intervals. ND: date not determinated.

Results

Viability of conidia from *B. bassiana*, *M. anisopliae*, and *P. lilacinum* isolates was higher than 89% at 24 h (Table 2). *B. bassiana* isolates caused infection and death of *H. betulinus* adults. Signs of infection were observed by external growing of fungal mycelia through the insect hosts cuticle; the fungal growth was first observed in intersegment membranes at the abdomen, then at mouthparts, antenna, and legs, where they sporulated and produced white conidia. The differences in mortality rate of *H. betulinus* controlled and treated for all 15 isolates used were highly significant ($F = 5.77$, df = 15, $P < 0.0001$). *B. bassiana* isolates caused > 50% mortality. The highest values were caused by isolate CEP 334, and the lowest was for CEP 347 (Table 2). The LT_{50} values ranged from 8.7 d (isolate CEP 340) to 13.5 d (isolate CEP 345) (Table 2).

M. anisopliae sensu lato isolates caused infection and death of *H. betulinus* after 7 d, and mycelia were observed all over the insect body with olive green color sporulation. The difference in mortality rate between controlled and treated insects was highly significant ($F = 16.82$, df = 2, $P = 0.0035$). The *M. anisopliae* isolates caused > 60% mortality, the maximum value being for CEP 350 and the lowest for CEP 349 (Table 2). The LT_{50} values ranged from 7.4 d (isolate CEP 349) to 7.9 d (isolate CEP 350) (Table 2).

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The seven isolates of *P. lilacinum* were not infective to *H. betulinus*. The differences in mortality rate of *H. betulinus* betwen controlled and treated insects were significant (F = 3.77, df = 7, P = 0.0133). *Purpureocillium lilacinum* isolates were not pathogenic. Because of this low mortality, it was not possible to calculate LT_{50} values (Table 2).

Discussion

In this study, *B. bassiana* and *M. anisopliae* sensu lato isolates presented high pathogenicity against *H. betulinus* adults. The different isolates of *P. lilacinum*, however, were not pathogenic. Hafez et al. (1994) reported that infection levels are the result of contact between a virulent strain and an insect cuticle that is susceptible to the fungus conidial germination and germ tube penetration. Finally, the pathogen can develop inside the insect body.

St. Leger et al. (1992) suggested that insects' susceptibility or resistance to a particular fungus can be determined by cuticle components at the beginning of the infection. Another aspect to be considered about the increase of pathogenicity is the *B. bassiana* production of extracellular enzymes in a nutritious culture medium (for example, proteases, lipases, and chitinases), all of which are included in host cuticle penetration and, consequently, in the infection by the fungus; their expression is influenced by cuticle composition and its own genesis in the culture medium (St. Leger et al. 1992, El Sayed 1993).

Pagliosa et al. (1994) reported similar results to ours when they evaluated *B. bassiana* CG 152; a mortality rate of 73.4% and a LT_{50} value of 9.4 d were obtained for adult *H. betulinus* under laboratory conditions. Shimazu et al. (2002) evaluated the pathogenicity of fungi *B. bassiana*, *M. anisopliae*, and *Paecilomyces* (= *Isaria*) sp. against the Cerambycidae *Anoplophora glabripennis* (Mots). The fungi were applied on larvae in a concentration of 1×10^7 conidia/mL. *B. bassiana* F0003 was the most infective isolate, causing 100% mortality with an LT_{50} value of 16 d.

Other previous reports of *Beauveria* sp. for control of the yerba mate pests *Thelosia camina* Sachus (Lepidoptera: Bombycidae) and *Hylesia* Hub. (Lepidoptera: Saturniidae) produced between 15.9 and 97% mortality when insects were exposed to 1×10^8 or 1×10^5 conidia/mL, respectively (Dalla Santa et al. 2009). Gomm et al. (2010) reported the efficacy of different dosages of the fungal formulation based on *B. bassiana* in the control of *H. betulinus* adults in field conditions. The treatments were 0, 2, 26, 50, 74, and 98 mL of the formulation based on *B. bassiana* CG 716 (Bovemax) with 1×10^7 conidia/mL. The doses of 50 and 98 mL were similar to each other and also were the only ones that were different from the control, though 50 mL has shown 33.26% of efficacy and the average time that the fungus took to kill the insect was 34.05 d. Leite et al. (2011) tested in laboratory several isolates of *B. bassiana*, *B. brongniartii*, *M. anisopliae* and *Paecilomyces* (= *Isaria*) sp. against *H. betulinus* adults. The concentration of fungal conidia applied was 3.5×10^7 conidia/mL in all cases. Mortality produced by *B. bassiana* varied between 66 to 100%, and their TL_{50} values varied from 9.8 to 26.4 d. These results were similar to ours, with a mortality rate between 51.1 and 86.3% and LT_{50} values between 9.6 and 13.6 d. However, Leite et al. (2011) observed that *M. anisopliae* and *B. brongniartii* caused 2.1 to 31.2% mortality and LT_{50} values from 17 to 25.8 d, differing from our results (mortality of 69.6 to 81.8% and LT_{50} from 7.4 to 7.9 d). In

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the previous report, *Paecilomyces* sp. produced 37.5 % mortality, whereas, in our study, the isolates of *P. lilacinum* were not pathogenic.

Our results indicate that different genera or species of entomopathogenic fungi have different pathogenicity. The median survival time can be attributed to various factors related to isolate infectivity and insect susceptibility. Virulence, infectivity, and pathogenicity are considered important properties of entomopathogens (Casadevall and Pirofski 1999, 2001; Thomas and Elkinton 2004). According to several authors preliminary records, entomopathogenic fungi infect insects to produce a large amount of secondary metabolites, including toxins attributed to pathogenicity, among which are low molecular weight compounds and other peptidic nature, as well as enzymes involved in the attack on the host (Pucheta et al. 2006, Borges et al. 2010. Molnar et al. 2010. Franco Chávez 2011, Rohlfs and Churchill 2011).

Our results demonstrate a pathogenic effect of *B. bassiana* and *M. anisopliae sensu lato* on *H. betulinus* adults under laboratory conditions. Further research is necessary to determine the effectiveness of *B. bassiana* and *M. anisopliae* sensu lato under field conditions and to examine its potential impact on non-target species.

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