Research Article

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Relationships between Host Insect, Enzymatic Activity and Virulence of Isolates of the Entomopathogenic Fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales)

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Abstract: Entomopathogenic fungi are important natural and biological control agents for many insects and other arthropods. The major mechanism driving the infection process is the secretion of hydrolytic enzymes such as proteases and chitinases. In this study, the effect of *in vivo* serial passage of *Beauveria bassiana* isolates through two different insect hosts (*Galleria mellonella* larvae and *Acanthoscelides obtectus* adults) and fungal enzymatic activity and virulence were evaluated. *In vivo* serial passage through the first insect host (*G. mellonella*) gradually increased the virulence of *B. bassiana* isolates, causing a three- to four-fold increase in mortality after six passages. The change of host from *G. mellonella* larvae to *A. obtectus* adults during passage caused a decline in *B. bassiana* virulence; however, successive passages in *A. obtectus* restored high virulence compared to initial passage in the new host. This manipulation also resulted in decreased protease activity but did not significantly affect chitinase activity. The results are discussed in relation to the chemical structure of insect cuticle and interactions in this host-pathogen system.

Key words: entomopathogenic fungi, virulence, protease, chitinase, insect host

Introduction

In nature, one of the most frequently observed examples of parasitism on insects are lethal infections caused by entomopathogenic fungi (Maddox 1994). Interactions occurring in entomopathogenic fungus-insect systems are highly complex and understanding dynamics on both sides of the system is crucial from ecological and practical contexts in the application of entomopathogenic fungi for biological control of pests (Meyling & Hajek 2010). Various studies have evaluated entomopathogenic fungi interactions with susceptible hosts illustrating the infection process of insect by fungi, as well as the adaptations and defence mechanisms of insects against fungal infection (Hajek & St. Leger 1994, Hajek 1997, Baverstock et al. 2010).

Entomopathogenic fungi infect insects by directly penetrating the external cuticle, thus the insect cuticle is the main barrier to initiation of the infection process. Penetration of the insect cuticle is the result of mechanical pressure and germ tube enzymatic activity of proteases and chitinases during germination of spores. These enzymes are thought to play a major role in pathogenesis (St. Leger et al. 1986, Wraight et al. 1990, St. Leger et al. 1991, Mondal et al. 2016). St. Leger et al. (1986) found that disintegration of the cuticle is the result of synergistic action of both types of enzymes. Proteases initiate the process of cuticle disintegration and then chitinases penetrate inside the insect body.

Mutual adaptation in a host-pathogen system is formed through co-evolutionary processes (Roy et al. 2006, Vilcinskas 2012). Variations in the susceptibility of insect species to fungal infection

and consequently observed differences in virulence may result from several factors, including differences in the structure and composition of the insect cuticle, the presence and activity of antifungal substances on the cuticle surface and in haemolymph, and the efficiency of cellular and humoural defence reactions of insects (Ortiz-Urquiza & Keyhani 2013). Some species of entomopathogenic fungi of the order Hypocreales, such as *Beauveria bassiana* (Bals.-Criv.) Vuill., have a wide range of hosts and are considered non-specialized pathogens (Inglis et al. 2001, Meyling et al. 2009). It seems, therefore, that they should quickly adapt to new hosts. The objectives of this study were to determine using *in vivo* passage method how quickly *B. bassiana* isolates adapt to the susceptible host, how quickly they adapt after host-switching, and how these factors affect virulence and enzymatic activity.

Materials and Methods

In the first stage of the study, various species of entomopathogenic fungi were isolated from soil using the Galleria bait method (Zimmermann 1986) and initial virulence (measured by insect mortality) was determined against first host *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae). Three *B. bassiana* isolates (B.b I, B.b II, B.b III) with low virulence were selected for passage through *G. mellonella*, so that any increase in virulence in subsequent passages could be recorded.

Passage through the host consisted of transferring the selected fungal isolate from the dead infected individuals to a healthy insect host. To obtain infectious material for the first passage, the initial isolates were cultured on Sabouraud's agar (SDA) for 14 days at 25°C. After sporulation, an aqueous suspension of spores with a density of $1x10^6$ spores/ml (in 0.05% Triton X) was prepared for insect inoculation. For each isolate, 6 passages were made in *G. mellonella* larvae (stage L3-L4) (the first host) and 6 passages in *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae) adults (the second host) according to the following inoculation and passage procedures. Ten adults of *A. obtectus* were placed in a test tube, flooded (immersed) with 10 ml of conidial suspension and immediately poured onto filter paper in a Buchner funnel (excess suspension was removed by vacuum). In order to protect the insects from escaping, the Buchner funnel was covered with a glass lid. However, ten larvae of *G. mellonella* were directly placed onto filter paper in a Buchner funnel, flooded with 10 ml of conidial suspension and within 2-3 seconds quickly drained off by suction (Inglis et al. 2012). After inoculation, the insects were carefully transferred and kept in Petri dishes lined with moist filter paper at 22-23°C. Insects immersed in sterile distilled water (with 0.05% TritonX) were used as controls. In the second passage, infectious material was obtained from dead insects covered with mycelium of *B. bassiana* from the first passage. To remove spores from insects after the first passage, insects were placed in a tube with a small amount of sterile distilled water and vortexed. The density of spores was determined in a haemocytometer (Thoma counting chamber). If necessary, the spore suspension was adjusted to obtain a density of $1x10^6$ spores/ml. Subsequent passages followed the same procedure. In each passage series, 30 insects (10 insects in 3 repetitions) were inoculated with each isolate.

Enzymatic activity

For the determination of the enzymatic activity, isolates were grown for 3 days (72 h) in Erlenmeyer flasks on a shaker (180 rpm, at 25° C) in liquid medium with a basic composition as follows (w/v): 2% dextrose, 0.5% peptone, 0.3% yeast extract, 0.1% K_2HPO_4, 0.2% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$ x $7H_2O$, 0.001% $FeSO_4$ (Hattori et al. 2005), in which 2% dextrose was replaced with 1% colloidal chitin (Sigma) as the carbon source to induce production of chitinases and proteases by the fungal isolates. The medium was inoculated with a spore suspension at a density of $1x10^6$/ml. The medium was then filtered (Whatman no. 1) to remove the mycelium, centrifuged, and the supernatant (filtrate) was used for further analyses.

Enzymatic activity was evaluated for each isolate after the first and sixth passages in *G. mellonella* and *A. obtectus*. Enzyme assays for each isolate were performed in triplicate. For all enzyme studies, the isolates were stored as a spore suspension in 10% glycerol at -20° C.

Protease assay

The protease assay was performed as described by Dhar & Kaur (2010) (according to the method of Kunitz 1947). Proteolytic activity was tested using casein as substrate. Proteolytic activity is expressed in universal units (U), where one unit of protease activity is defined as the amount of enzyme that produced 1 μmol of tyrosine per 1 min.

Chitinase assay

Chitinolytic activity was determined according Nahar et al. (2004) using colloidal chitin as the

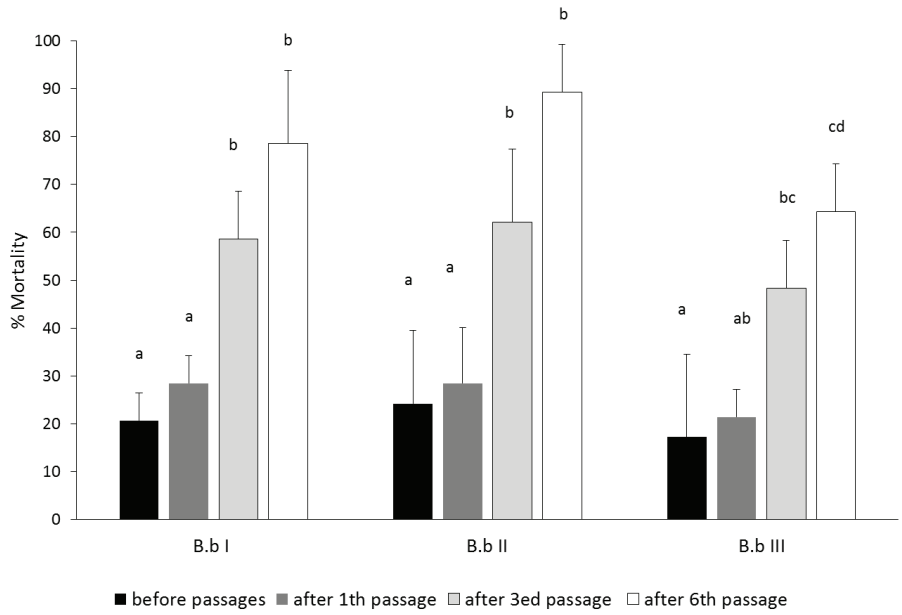

Fig. 1. Mortality of *Galleria mellonella* larvae induced by *Beauveria bassiana* isolates (B.b I, B.b II, B.b III) before passage, and after the 1th, 3rd and 6th passage in *G. mellonella* larvae (means with the same letter are not significantly different according to Duncan's test at 0.05).

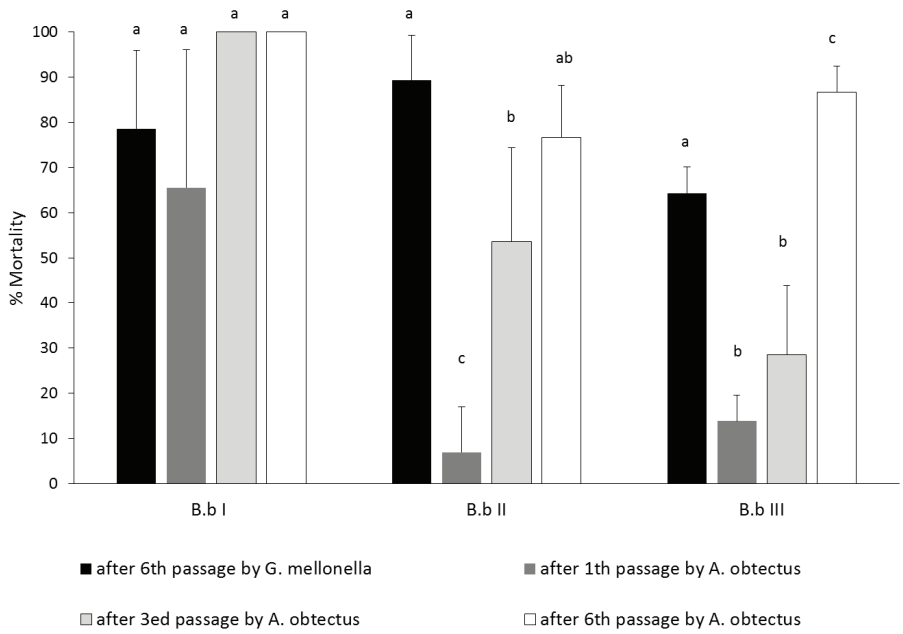

Fig. 2. Mortality of *A. obtectus* adults induced by *Beauveria bassiana* isolates (B.b I, B.b II, B.b III) following host change from *Galleria mellonella* larvae to *Acanthoscelides obtectus* (means with the same letter are not significantly different according to Duncan's test at 0.05).

substrate. Chitinolytic activity is expressed in universal units (U). One unit of chitinase activity is defined as the amount of enzyme that produced 1 µmol GlcNAc (N-acetylglucosamine) per 1 min.

Statistical analysis

Insect mortality was calculated as percent mortality corrected with natural mortality using Abbott's formula. Analysis of variance (one-way ANOVA) or Kruskal-Wallis H Test was performed to compare differences in virulence and enzyme activity among different *B. bassiana* isolates after successive passages and significant differences among means were compared using Duncan's test (α = 0.05). The data were statistically analysed using Statistica ver. 12.0 software.

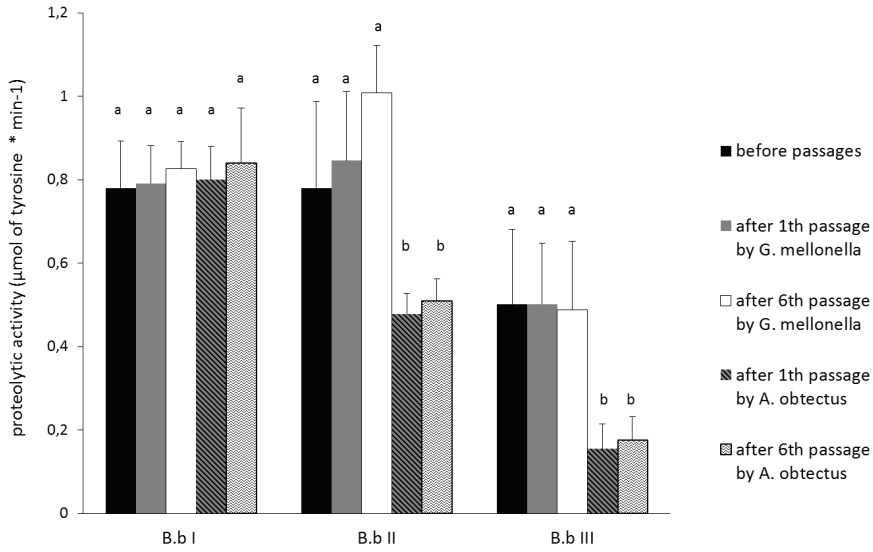

Fig. 3. Proteolytic activity of *Beauveria bassiana* isolates (B.b I, B.b II, B.b III) before passage, after the 1th and 6th passage in *Galleria mellonella* larvae, and after the 1th and 6th passage in *Acanthoscelides obtectus* adults (means with the same letter are not significantly different according to Duncan's test at 0.05).

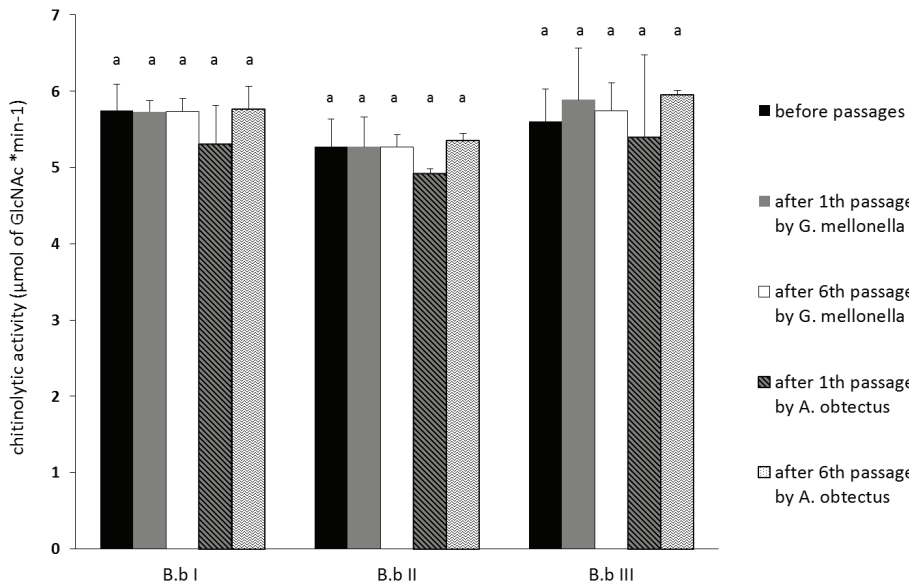

Fig. 4. Chitinolytic activity of *Beauveria bassiana* isolates (B.b I, B.b II, B.b III) before passage, after the 1th and 6th passage in *Galleria mellonella* larvae, and after the 1st and 6th passage in *Acanthoscelides obtectus* adults (means with the same letter are not significantly different according to Duncan's test at 0.05).

Results

Prior to passages through the first host (*G. mellonella* larvae), initial insect mortality from the three *B. bassiana* isolates was similar. Isolate B.b I caused 20.7% mortality, while B.b II and B.b III caused 24.2% and 17.3% mortality, respectively (Fig. 1). After the first passage, the *G. mellonella* larval mortality slightly increased. Subsequent passages significantly increased the virulence, and after 6 passages of the B.b I isolate, mortality reached 78.60% ($F_{(3, 8)} = 20.667$, $p<0.05$), for B.b II, mortality increased to 89.3% ($F_{(3, 8)} = 14.203$, $p<0.05$), and for Bb III, mortality increased to 64.3% ($F_{(3, 8)} = 6.397$, $p<0.05$) (Fig. 1).

The change in the host insect from *G. mellonella* larvae to *A. obtectus* during serial passages caused a significant decrease in insect mortality in isolates B.b II and B.b III after the first passage through the new host. For isolate B.b I,

insect mortality decreased by only 13% (H test $_{(3, 12)}$ = 5.5456, p = 0.1359), while for B.b II and B.b III, insect mortality decreased by more than 80% ($F_{(3, 8)}$ = 19.188, p<0.05) and 50% ($F_{(3, 8)}$ = 36.100, p<0.05), respectively (Fig. 2).

Subsequent passages of *B. bassiana* isolates in *A. obtectus* increased insect mortality to the same level of mortality observed after the sixth passage in *G. mellonella* (Fig. 2). Maximum mortality caused the B.b I isolate in the new host was achieved after the 3rd passage, but the greatest increase in insect mortality occurred in the B.b III isolate, from 13.8% (after the first passage) to 86.7% (after the sixth passage) (Fig. 2).

The proteolytic activity of the initial *B. bassiana* isolates (prior to passages through *G. mellonella*) was similar, with 0.78 U/ml for isolates B.b I and B.b II and 0.5 U/ml for isolate B.b III. After six passages in *G. mellonella*, no significant increase in proteolytic activity was observed (Fig. 3). After a host change from *G. mellonella* to *A. obtectus*, a significant decrease in proteolytic activity of B.b II ($F_{(4, 10)}$ = 8.714, p<0.05) and B.b III ($F_{(4, 10)}$ = 5.655, p<0.05) after the first passage was observed, whereas isolate B.b I proteolytic activity did not change with the new host ($F_{(4, 10)}$ = 0.186, p = 0.941). Subsequent passages in *A. obtectus* did not increase the proteolytic activity of B.b II and B.b III isolates (Fig. 3).

Chitinolytic activity in the initial *B. bassiana* isolates were similar prior to passage in *G. mellonella* (Fig. 4), as observed for proteolytic activity. After serial passages both in *G. mellonella* larvae and after changing the host to *A. obtectus* adults, chitinolytic activity was comparable to initial isolate activity for all *B. bassiana* isolates (B.b I $F_{(4, 10)}$ = 1.136, p = 0.394, B.b II $F_{(4, 10)}$ = 1.70, p = 0.344, and B.b III $F_{(4, 10)}$ = 0.403, p = 0.802) (Fig. 4).

Discussion

The process of insect infection by entomopathogenic fungi begins with the attachment of the spores to the surface of the insect cuticle. The insect cuticle is thus the first and a major barrier in the initiation and completion of the fungal life cycle (Wraight et al. 1990, St. Leger et al. 1991). The next critical step in the infection process is cuticle penetration by germinated spores, which is the result of the combined action of mechanical force and the enzymatic action of chitinases, proteases, and lipases secreted by the fungus. The production of cuticle-degrading enzymes is thought to be an important factor in determining virulence in entomopathogenic fungi (St. Leger et al. 1986, Gupta et al. 1994, Zare et al. 2014, Mondal et al. 2016); however, recent studies have shown that different species, and isolates can vary in the production of cuticle-degrading enzymes (Dhar & Kaur 2010, Revathi et al. 2011, Mohammadbeigi 2013). According to Zare et al. (2014), these variations in the secretion of enzymes may be responsible for the differences in virulence observed in different species or strains of entomopathogenic fungi. In the current study, despite the increase in virulence, I observed no increase in proteolytic and chitinolytic activity against hosts.

My results agree with others reporting a positive effect of passage through the host on virulence of entomopathogenic fungi (Hayden et al. 1992, Qesada-Moraga & Vey 2003, Butt et al. 2006). The *in vivo* passage technique used in this study is the recommended protocol for restoring activity and increasing the virulence of entomopathogenic fungi after subculture on artificial media (Aizawa 1971, Ignoffo et al. 1982, Song & Feng 2011). Many authors have reported that the subculture of entomopathogenic fungi in artificial media can cause phenotypic changes, including colony colour and morphology, and may be associated with a decrease in sporulation and virulence (Butt et al. 2006, Mohammadbeigi 2013, Thaochan & Chandrapatya 2016). This technique is also widely used in various biological control approaches, because it increases virulence and reduces the total time needed to suppress target pest populations (Adames et al. 2011).

The purpose of using this method in the present study was to observe how the pathogen evolves over passage among individuals and species and how quickly the pathogen adapts to a new host. This method at least partially reflects what occurs under natural conditions in insect populations during direct (horizontal) or indirect transmission. In the current study, subsequent passages through the insect host gradually increased the virulence of *B. bassiana* isolates, causing a three- to four-fold increase in *G. mellonella* mortality after six passages, and a nearly six- to eleven-fold increase in *A. obtectus* mortality. The change of host from *G. mellonella* larvae to *A. obtectus* adults caused a significant decrease in *B. bassiana* virulence (except isolate B.b I); however, successive passages in *A. obtectus* restored high virulence compared to initial passage in the new host. Quesada-Moraga & Vey (2003) reported that *B. bassiana* strain 90/2-Dm originally isolated from *Dociostaurus maroccanus* Thunberg increased virulence and secretion

of toxins after only two passages in *Locusta migratoria* Linnaeus. Results from the same study also show that host change leads to rapid adaptation to the alternative host. This indicates high plasticity of *B. bassiana* different strains and the relatively fast rate of adaptation to new hosts. My results supported the hypothesis that entomopathogenic fungi such as *B. bassiana* that have a wide range of hosts adapt quickly to new hosts. This adaptation allows them to spread and survive in the environment.

The antagonistic relationship between insect host and entomopathogenic fungi means that both host and pathogen must constantly adapt to the new system. Insect hosts must improve their defence mechanisms against fungal pathogens, and pathogen must "keep up" with the changing host by increasing virulence or developing other adaptations for survival. There is ample evidence that the insect cuticle produces fungistatic compounds that inhibit spore germination a as a defence against infection (Gross et al. 2008, Gołębiowski et al. 2013). After breaking the cuticle barrier by germinating spores, insects switch on cellular and humoural immune responses (Gillespie et al. 1997, Wojda et al. 2009). There are also known examples of non-specific morphological and behavioural defence mechanisms to avoid fungal infections (Baverstock et al. 2010). The insect cuticle is a very complex, fibrous structure consisting of a thin epicuticle and a thicker inner procuticle that consists of a protein matrix containing chitin, lipids and microfibrils, usually arranged in layers (Ortiz-Urquiza & Keyhani 2013). Insect cuticle can differ in structure depending on the insect species or the stage of development. The species and developmental stage may have distinct biochemical composition and may have different protein profiles (Andersen et al. 1995, Cox & Willis 1987, Kramer et al. 1988). This highlights the need for studies of virulence and enzymatic activity and the rate of adaptation of the different strains and isolates of *B. bassiana*, as they may differ depending on the hosts used in laboratory experiments, particularly if they belonging to different insect orders whose cuticle construction may vary (e.g., Lepidoptera and Coleoptera). *Galleria mellonella* larvae have relatively thin layers of cuticle, called "soft" cuticle, while *A. obtectus* has "hard" cuticle. Boguś et al. (2007), during histological examination of *G. mellonella* larvae exposed to highly pathogenic *Conidiobolus coronatus* (Constantin) Batko (Entomophthorales), revealed that the cuticle of *G. mellonella* larvae was approximately seven and four times thinner than the cuticle of *Calliphora vicina* Robineau-Desvoidy (Diptera) and *Dendrolimus pini* Linnaeus (Lepidoptera) larvae, respectively. In the same study, each of the insects tested also showed different defence strategies against fungal infection (Boguś et al. 2007). These differences in the structure and chemical composition of insect cuticle may reflect differences in the susceptibility of insects to fungal infections. In the current study, I found that serial passage of *B. bassiana* in the first host, *G. mellonella* larvae, two highly virulent isolates towards this host (B.b II and B.b III) caused low mortality in *A. obtectus* adults. Isolate B.b I did not react to the host change, and only slightly reduced the mortality of the new host.

Studies investigating how the manipulation of hosts (exchange of host species during subsequent passages) affects pathogen virulence and enzymatic activity are scarce in the literature (Quesada-Moraga & Vey 2003, Vandenberg & Cantone 2004). In the case of proteolytic activity, the change in host from *G. mellonella* to *A. obtectus* resulted in both a decrease in virulence and secretion of proteolytic enzymes (for two of the three tested isolates), while there was no relationship between the change of host and chitinolytic activity. *In vivo* passages did not affect the chitinolytic activity of any *B. bassiana* isolates in either host compared to initial isolates. The results obtained by Vandenberg & Cantone (2004) also demonstrate that manipulation of host species during *in vivo* passages can affect the virulence another species of entomopathogenic fungus, *Paecilomyces fumosoroseus* (current name *Isaria fumosorosea* Wize), as well as other traits of this fungus. Their results also showed the intraspecific variability and phenotypic plasticity of strains of *P. fumosoroseus*, because despite the changes in virulence, conidiation, and speed of conidia germination, no changes in banding pattern were observed for any strain using 14 primers in RAPD-PCR (Vandenberg & Cantone 2004).

Recently, molecular studies of entomopathogenic fungi have provided strong evidence that pathogenesis is controlled by the genes responsible for the secretion of insect cuticle-digesting enzymes (Fang et al. 2005, Enkerli et al. 2009, Boldo et al. 2010). However, there are also examples that non-molecular mechanisms such as better conidia adhesion, faster rate of germination, or increased antimicrobial substances on the insect cuticle, can also play a role (St. Leger et al. 1991, Crespo et al. 2002, Adames et al. 2011).

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