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Genetic Variation in a Chitinase Gene of *Beauveria bassiana*: Lack of Association Between Enzyme Activity and Virulence Against *Hypothenemus hampei*

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Abstract: Like other entomopathogenic fungi, *Beauveria bassiana* produces enzymes that degrade cuticle for penetration in the host at the time of infection. Chitinases are considered important enzymes for chitin hydrolysis, one of the main insect exoskeleton components. In this study, polymorphism in *B. bassiana* chitinase gene was analyzed by PCR-RFLP and compared with the enzymatic activity and mortality caused on adults of coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae). Thirty *B. bassiana* isolates obtained from different insect species and geographic origins were used. The activity of chitinases was not directly related with the mortality rate of each strain, emphasizing the hypothesis that the virulence is multifactorial. The chitinase gene analyses showed low variability between isolates, as only four isolates presented polymorphism. Therefore it was not possible to correlate the polymorphism with virulence and chitinolitic activity. Lack of association between chitinase gene polymorphism and enzyme activity suggests that the polymorphic region studied may not be involved in enzymatic activity of this gene. Further, lack of association between enzyme activity and virulence suggests that there may be other enzymes and factors that could contribute to infection ability. No association between polymorphism in chitinase gene with that of geographic region or origin was observed.

Key words: *Beauveria bassiana*, chitinase, polymorphism, PCR-RFLP, virulence, *Hypothenemus hampei*

INTRODUCTION

Beauveria bassiana (Bals.) Vuill. (Hyphomycetes) is an opportunistic and cosmopolitan deuteromycete (Alves, 1998) that has been extensively used as a biocontrol agent against many insect species. This fungus has been largely used for the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) control in many countries around the world (Gaitan *et al*., 2002). The virulence analysis of 61 *B. bassiana* strains, against this species, have shown variable rates of mortality, ranging from 3.3 to 83.3% (Neves and Hirose, 2005). The different virulence levels may be related with the production of enzymes and toxins involved at the infection process (Tanada and Kaya, 1993; Alves, 1998).

The mode of infection is through the exoskeleton, which is the first barrier for the insect's protection (Clarkson and Charnley, 1996). As the exoskeleton is mainly composed by proteins and chitin, proteases and chitinases should be considered the main enzymes involved in the fungi infection process (St. Leger *et al*., 1996; Kang *et al*., 1998; Nahar *et al*., 2004).

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Although it has been demonstrated that proteases initiate cuticle degradation creating a more efficient environment for the chitinase action (St. Leger *et al*., 1986), *in vitro* studies have shown that proteases and chitinases act synergistically at the cuticle solubilization process (St. Leger *et al*., 1996). In spite of proteases being important virulence factors, this enzyme should not be considered as the single essential factor for the virulence, once *B. bassiana* strains unable on producing this enzyme, showed only a reduction in the infection ability (St. Leger and Joshi, 1997). Chitinases hydrolyse chitin at the β-1,4 glycosidic bonds liberating the N-acetylglucosamine residues (Tharanathan and Kittur, 2003). Chitinase activity during the infection process seems to be extremely important once the chitin composition in the insect exoskeleton reaches 20 to 50%, of the dry weight (Chapman, 1998).

Many studies investigating correlation between genetic polymorphisms of entomopathogenic fungi strains and geographic origin or insect host have been reported (Maurer *et al*., 1997; Coates *et al*., 2002; Gaitan *et al*., 2002; Muro *et al*., 2003; Wang *et al*., 2003), but there are few studies focusing on virulence candidate genes. Wang *et al*. (2003) verified DNA polymorphisms, using the PCR/RFLP method, at the protease (PR1) gene from *B. bassiana* and found a large amount of variability at this gene that was more related with the geographic origin than with the insect host.

Considering that sequence variation at virulence-related genes seems to occur more frequently than at other loci (Internal Transcribed Spacer, for instance) (Muro *et al*., 2003), the goal of this study was to verify the incidence of polymorphisms at the chitinase gene in 30 isolates of *B. bassiana* and try to correlate this polymorphisms with *in vitro* chitinase activity and virulence against adult coffee berry borer, *H. hampei*.

MATERIALS AND METHODS

Fungal Strains

Thirty *B. bassiana* strains (Table 1) stored at the Entomopathogen Collection in the Department of Agronomy at State University of Londrina were investigated in this study. Biochemical and molecular analysis were conducted at the Biochemistry and Biotechnology Department of the Londrina State University, Paraná, Brazil.

Fungal Growth Conditions

B. bassiana strains used in this study had been previously bioassayed in the coffee berry borer (*H. hampei*) (Neves and Hirose, 2005). Strains were initially inoculated into solid culture medium (w/v: 2% agar, 1% glucose, 0.5% yeast extract, 0.158% $NaNO_3$, 0.105% $Na_2HPO_4.7H_2O$, 0.1% KCl; 0.06% $MgSO_4.7H_2O$, 0.036% KH_2PO_4 and 0.05% streptomycin sulfate) (Alves, 1998), grown at 25°C, for 10 days, 12 h photofase and then inoculated in coffee berry borer adults as described by Neves and Hirose (2005). After conidiogenesis on insect, each strain was inoculated into solid culture medium and conidia used for preparation of a 10^8 conidia mL^{-1} suspension that was inoculated in liquid culture medium. Submerged cultures were carried out at 28°C, 150 rpm by addition of 1% of the conidia suspension. The crude extract (CE) obtained by 5 days cultures was filtrated using Whatman No.1 filter and was dialized into 12 kDa molecular cutoff membrane against 5 mM pH 5.0 acetate buffer at 4°C for 24 h.

Chitinase Assay

Chitinase activity was determined using the protocol described by Nahar *et al*. (2004) with slight modifications: 0.5 mL aliquots of CE were incubated with 1mL of 1% colloidal chitin (Practical Grade, Sigma) (Kang *et al*., 1999) prepared in 50 mM acetate buffer pH 5.5 at 45°C. After 1 h, enzyme reaction was interrupted at 100°C for 1 min. Residual chitin was separated by centrifugation at 1,000 g for 15 min and the supernatant was used for determination of N-acetylglucosamine using the method described by Reissig *et al*. (1955). One unit of enzyme activity was defined as micrograms of N-acetylglucosamine released per mL of CE at reaction conditions.

Table 1: Host and geographic origin of *Beauveria bassiana* isolates used to verify enzymatic activity and polymorphism in chitinase gene

Isolate	Insect host	Family	Origin
CB102	Soil		SP-Brazil
CB66	*Hypothenemus hampei*	Coleoptera: Scolytidae	SP-Brazil
CB97	*Cosmopolites sordidus*	Coleoptera: Curculionidae	Unknown
CG82	*Diabrotica speciosa*	Coleoptera: Crysomelidae	TU-Argentina
CG425	*Rhammatocerus schistocercoides*	Orthoptera: Acrididae	MT-Brazil
447-ESALQ	*Solenopsis invicta*	Hymenoptera: Formicidae	MT-Brazil
CG481	*Diabrotica speciosa*	Coleoptera: Crysomelidae	BA-Brazil
CG26	Species not identified	Coleoptera	DF-Brazil
CG432	Species not identified	Heníptera: Membracidae	RN-Brazil
CG458	*Anthonomus grandis*	Coleoptera: Curculionidae	PR-Brazil
CG138	*Cosmopolites sordidus*	Coleoptera: Curculionidae	PE-Brazil
CG368	Soil		PE-Brazil
CB64	*Hypothenemus hampei*	Coleoptera: Scolytidae	RO-Brazil
UEL54	*Diabrotica* sp.	Coleoptera: Crysomelidae	PR-Brazil
UEL08	*Hypothenemus hampei*	Coleoptera: Scolytidae	PR-Brazil
UEL04	*Hypothenemus hampei*	Coleoptera: Scolytidae	PR-Brazil
CG375	*Colaspis* sp.	Coleoptera: Crysomelidae	DF-Brazil
CB95	*Solenopsis invicta*	Hymenoptera: Formicidae	MT-Brazil
UEL55	Species not identified	Hemiptera	PR-Brazil
UEL07	*Hypothenemus hampei*	Coleoptera: Scolytidae	PR-Brazil
CB74	*Lyssorhoptus oryzophilus*	Coleoptera: Curculionidae	Japan
CB82	*Cosmopolites sordidus*	Coleoptera: Curculionidae	Unknown
CG166	Species not identified		PR-Brazil
CG71	*Diatraea saccharalis*	Lepidóptera: Pyralidae	PE-Brazil
CG25	*Anticarsia gemmatalis*	Lepidóptera: Noctuidae	DF-Brazil
CG245	Species not identified	Hymenoptera: Megachilidae	GO-Brazil
CB35	*Cosmopolites sordidus*	Coleoptera: Curculionidae	BA-Brazil
CB47	*Anthonomus grandis*	Coleoptera: Crysomelidae	SP-Brazil
UNI4	*Alphitobius diaperinus*	Coleoptera: Tenebrionidae	PR-Brazil
CB17	*Anthonomus grandis*	Coleoptera: Curculionidae	SP-Brazil

DNA Extraction

The mycelium was obtained by filtration of the liquid cultures, washed with sterile water and dried using sterile absorbent paper. It was then ground into a fine powder in liquid nitrogen and the DNA was extracted from this powder using the protocol previously described by Bogo *et al*. (1996). Pelleted DNA was resuspended in 60 μL of ultrapure water and stored at 4°C. The quality and quantity of extracted DNA samples was monitored routinely by electrophoresis of the material in a 1% agarose gel in TBE buffer (89 mmol L^{-1} Tris borate, 89 mmol L^{-1} boric acid, 2 mmol L^{-1} EDTA; pH 8), stained with ethidium bromide and observed under UV light. Quantification was performed through comparison with known dilutions of lambda phage DNA.

PCR Reaction

A pair of primers were designed from a 1047 bp nucleotide sequence, corresponding to the chitinase (Bbchit1) gene from B. bassiana that was retrieved from the GenBank (Access Number AY145440) (Fang *et al*., 2005). Primers were designed (Chit 1F 5′-GCTCGCAACATACCAATC-3' and Chit 1R 5′-GTCGCCAAATGTCCAATTC-3') using the Gene Runner (Hastings Software, Inc). The PCR reactions were carried out in a final volume of 10 μL with the following composition: 5 ng of the DNA, 1 μL of the 10X reaction buffer (200 mmol L^{-1} Tris-HCl pH 8.4; 500 mmol L^{-1} KCl), 200 μmol L^{-1} of each dNTP, 10 pmol of each primer, 1.5 mmol L^{-1} of $MgCl_2$, 1U of Taq DNA polymerase. The temperature program was composed of an initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. All reactions were repeated at least twice, always including both negative (DNA-free) and positive

controls. The success of the reaction was confirmed by running the product in 1% agarose gel stained with ethidium bromide and visualized under UV light. Pearson's correlation coefficient were calculated to study the association between chitinase activity and virulence of all lineages against *Hypothenemus hampeii*.

PCR/RFLP Analysis

Restriction enzyme screening was done using the pDRAW32 (AcaClone) based on the same sequence used for the primer design. Nine enzymes capable of cutting the PCR fragment into two or three fragments: BglI (518/517 bp), BseYI (899/136 bp), DrdI (593/319/123 bp), HgaI (726/216/93 bp), FokI (776/199/60 bp), AflIII (579/344/112 bp), BtgI (546/418/71 bp), BstF5I (763/212/60 bp) and KasI (528/414/93) were selected. Aliquots (10 μL) of PCR products were digested for 4 h at 37°C with the exception of BstF5I that was incubated at 65°C. Digestion reactions were carried out in a 15 μL final volume with 1.5 μL of 10X reaction buffer and 1.5 μL of BSA (100 μg mL^{-1}) according to the manufacturer's instructions. The restriction fragments were analyzed by electrophoresis in 1 X TBE buffer with 3% agarose gels and detected by UV fluorescence after ethidium bromide staining. The 1 Kb plus DNA ladder (Invitrogen) was used as a molecular size marker. The occurrence of polymorphisms was compared with the *in vitro* chitinase production and virulence against *H. hampei* in all of the 30 *B. bassiana* isolates.

RESULTS AND DISCUSSION

The highest chitinase activity (1510.8 U mL^{-1}) was detected for isolate CB95 but which only caused 36.7% of mortality. On the other hand, the most virulent isolate, causing the highest mortality (CB102: 83.3%), presented low chitinase activity, 46.4 U mL^{-1}. Thus the *in vitro* chitinase activity was not directly related to the mortality rates in *H. hampei* (r = -0.122) that was previously described by Neves and Hirose (2005) (Table 2).

Using the above-described PCR protocol, a single product of approximately 1.035 Kb was amplified from all of the 30 isolates of *B. bassiana*. This corresponded to the estimated size based on the previously GenBank deposited sequence of chitinase gene. Enzymatic digestions of this PCR product generated the expected band patterns with all restriction enzymes. Three (DrdI, FokI and KasI) of the 9 studied enzymes showed polymorphism in 4 of the 30 isolates analyzed.

Figure 1 shows the polymorphic patterns observed in these isolates: CG245, UEL04, CB47, CG138. The chitinase gene has two restriction sites for DrdI and digestion of the 1.035 Kb PCR product was expected to generate three fragments of approximately 593, 319 and 123 bp. However, the restriction of the sequence amplified from strain CG245 showed only two fragments (300 and 735 bp), suggesting the loss of a restriction site in this strain. The mortality rate caused by CG245 was 8% and can be considered one of the lowest, although the chitinase activity was considerably high: around 400 U mL^{-1}. This data suggest that the mutation was not associated with virulence level on insects, as isolates causing high (CB102) and low (CB47) mortality rates in insects showed the same restriction pattern for this gene. Any association would be revealed by the chitinase activity and also polymorphic profile.

The PCR product of the isolates UEL04 and CB47, when digested with FokI, showed a different pattern from that expected, with the 776 bp original fragment digested in two with approximately 700 and 76 bp. The KasI digestion of the CG138 PCR fragment produced Four fragments (407, 277, 255 and 96 bp). This restriction pattern suggests a new mutational site for this enzyme as the expected 528 bp fragment was cut in two of 277 and 255 bp. The mortality caused by UEL04 was around 40% while the strain CB47 caused only 3% death; this observation shows a non-association between these polymorphic profiles and the mortality rates against *H. hampei*. The *in vitro* enzymatic activity also

Table 2: Mortality on adult coffee berry borer (*Hypothenemus hampei*) (Neves and Hirose, 2005) and chitinase activity of the 30 *Beauveria bassiana* (2.5×10^7 conidia mL^{-1}) isolates

Isolate	Mortality (%)	Chitinase activity (U mL^{-1})
CB102	83.33	46.363
CB66	80.56	380.220
CB97	77.78	31.126
CG82	76.67	9.255
CG425	73.33	34.465
447-ESALQ	73.33	26.391
CG481	69.44	422.995
CG26	66.67	98.485
CG432	63.33	25.510
CG458	56.67	140.890
CG138	50.00	444.960
CG368	50.00	136.435
CB64	50.00	0.000
UEL54	47.22	28.175
UEL08	46.67	599.380
UEL04	44.44	200.095
CG375	40.00	415.500
CB95	36.67	1510.835
UEL55	36.67	27.170
UEL07	30.00	179.910
CB74	23.33	303.500
CB82	20.00	23.786
CG166	16.67	676.290
CG71	16.67	22.760
CG25	13.33	0.000
CG245	8.00	405.685
CB35	5.56	11.695
CB47	3.33	77.685
UNI4	NA	26.865
CB17	NA	112.620

NA: Not available

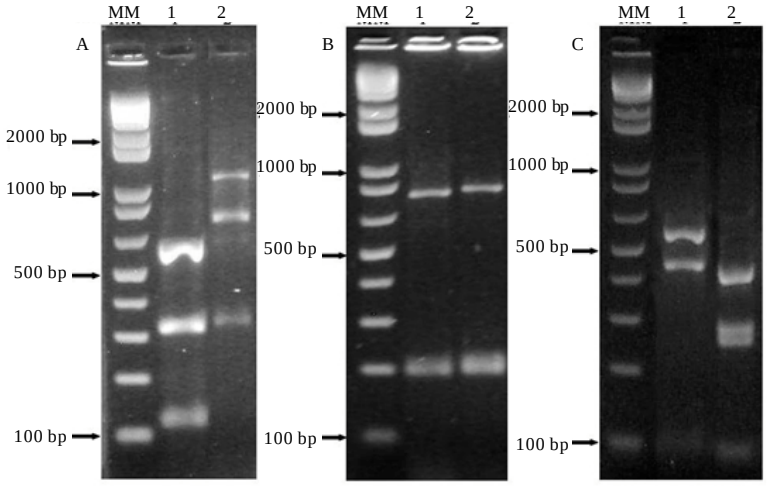

Fig. 1: Electrophoretic profiles of the digested chitinase gene in *B. bassiana*. (A) strain CG245 digested with DrdI (lane 2), (B) strain UEL04 digested with FokI (lane 2) and (C) strain CG138 digested with KasI (lane 2). Lanes 1 in all figures shows the restriction profile obtained in other strains. MM indicates the DNA molecular weight marker 1 Kb Plus DNA ladder

did not show any association with the restriction patterns (UEL04: 200.095 U mL^{-1} and CB47: 77.685 U mL^{-1}). Similarly, CG138 showed an intermediate mortality rate (50%) and chitinase activity (444.96 U mL^{-1}) which lead us to infer that this mutation was not related with the ability to infect and kill the insect host. No association was observed between geographic origin or insect host and the genetic variation. The isolates UEL04 and CB47 presented the same polymorphic pattern in spite of being isolated from different insects at different geographic regions (*H. hampei* from Paraná and *A. grandis* from São Paulo, respectively). Likewise, the strain CG138 isolated from *Cosmopolites sordidus* showed a different restriction pattern when compared with other isolated from the same species (CB97 e CB35) and in the case of CG138 and CB35, at the same geographical region (Brazilian Northeast). The strain CG245 isolated from a Hymenoptera from the Goiás State showed a different profile from others (CB95 and 447-ESALQ) isolated from the same host. However, as this isolate was unique to this area, further sampling is needed to verify if some local evolutionary forces were influencing the genetic profile. The lack of association between geographical region or host preference and genetic variation has been previously related (Muro *et al*., 2003; Gaitan *et al*., 2002; Coates *et al*., 2002), using different methodologies, like the Internal Transcribed Spacer sequencing, as well as RFLP and RAPD analysis.

Wang *et al*. (2003) found association between the protease gene variation and microsatellite molecular markers with geographical locations of the isolates, while Maurer *et al*. (1997) suggests that the insect host is the major selection factor on the evolutionary process of *B. bassiana*. The absence of a visible association between activity of chitinase, which is one of the pathogenicity-related factor, RE polymorphisms and virulence of *B. bassiana* on adults of the coffee borer (*H. hampei*) suggests that several genes act together in the process of infection/colonization. It is possible that alterations in the chitinase nucleotide sequence are not related with the fungal efficiency in infecting insects and that the virulence, because of being defined by a combination of several factors, is controlled by other regulatory genes or post-translational modifications of the enzymes and may be some determinative virulence factors are less efficient in less virulent isolates.

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