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Boophilus microplus Infection by *Beauveria amorpha* and *Beauveria bassiana*: SEM Analysis and Regulation of Subtilisin-like Proteases and Chitinases

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Abstract. *Beauveria bassiana* is a well-known broad-range arthropod pathogen which has been used in biological control of several pest insects and ticks such as *Boophilus microplus*. *Beauveria amorpha* has both endophytic and entomopathogenic characteristics, but its capacity for biological control has still not been studied. During the processes of host infection, *B. bassiana* and *B. amorpha* produce several hydrolytic extracellular enzymes, including proteases and chitinases, which probably degrade the host cuticle and are suggested to be pathogenicity determinants. To access the role of these enzymes during infection in the tick *B. microplus*, we analyzed their secretion during fungus growth in single and combined carbon sources, compared to complex substrates such as chitin and *B. microplus* cuticle. Chitin and tick cuticle-induced chitinase in both fungus and protease was induced only by tick cuticle. SEM analysis of *B. amorpha* and *B. bassiana* infecting *B. microplus* showed apressorium formation during penetration on cattle tick cuticle.

Beauveria bassiana-based mycoinsecticides have been developed and registered worldwide for control of agricultural pests [17, 29], usually being applied in the fields as a conidial spray [19]. This fungus infects a wide range of insects such as thrips, beetles [13], flies [20], and several species of ticks [19, 21] and is commonly found in nature [7]. The tick *Boophilus microplus* is a bovine ectoparasite that causes significant economic losses in herds of tropical and subtropical areas. It transmits diseases and causes reduction in milk and meat yield and leather production. The necessity of tick control represents significant investment and the present technology is based on the use of synthetic chemical products. However, the ability of *B. microplus* to develop resistance to acaricides, the demands of consumers for chemical free foods, and the negative environmental effects of acaricides call for the development of alternative strategies. Therefore, efforts to develop alternative methods, such as biological control of ticks using filamentous fungi, chiefly *Metarhizium anisopliae* [12] and *B. bassiana* [23], have been pursued.

To transpose the cuticle, the main host barrier, entomopathogenic fungi utilize a combination of mechanical and enzymatic mechanisms, and secretion of proteases is believed to be an important pathogenic factor for fungal attack on cuticle [28].

The best-understood model of a fungal determinant of entomopathogenicity is based on *M. anisopliae*, the subtilisin-like endoprotease designated Pr1 [30]. This enzyme is adapted to extensively degrade insects' cuticular proteins [28] and has been ultrastructurally located in the host cuticle during the early stages of penetration [15]. The occurrence of natural variability in the production of cuticle-degrading proteases among

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isolates of *M. anisopliae* after growth on cuticular and noncuticular substrates has being investigated [3, 22]. Field studies have demonstrated that *B. bassiana* also colonizes corn, endophytically [5].

Beauveria amorpha has both endophytic and entomopathogenic characteristics, but its capacity for biological control has not been studied. To help understand the role of proteases and chitinases in *B. microplus* cuticle penetration, it is desirable to determine how their synthesis is regulated in these two fungal models. Knowledge of how protease and chitinase production is regulated could be highly relevant to understanding the pathogenic process. Therefore, this work aims to analyze the production of both enzymes by *B. bassiana* and *B. amorpha* and to investigate the infection process in *B. microplus* by scanning electron microscopy (SEM), to identify possible variations that may be relevant for tick biocontrol and for the development of commercial formulations.

Materials and Methods

Organisms and culture conditions. *Beauveria bassiana* strain CG166, originally isolated from *Schrius* sp. (Curitiba/PR, Brazil), was supplied by Empresa Brasileira de Pesquisa Agropecuária (Embrapa/Cenargen, Brasília/DF, Brazil). *Beauveria* B95 was isolated from *Zea mays* leaves by Dr. Ida Chapaval Pimentel (Universidade Federal do Paraná/UFPR, Brazil). DNA from this endophytic isolate (*Beauveria* B95) was characterized by sequencing 5.8S rDNA, ITS-1, and ITS-2, using the primers ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′), which is specific for higher fungi, and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′), which is a universal primer. The purified PCR products (GFX PCR DNA and band purification kit; GE Healthcare) were then sequenced in both directions using the ITS-1 and ITS-4 primers and the sequences compared to the GenBank database using the BLASTn program [1], which showed that this isolate is closer to *Beauveria amorpha* than to *Beauveria bassiana*. Conidia suspensions were prepared in 0.01%. Tween 80 solution from fungi grown on Sabouraud dextrose agar plates. After washing in H_2O, conidia suspensions were maintained in 10% glycerol at a concentration of 10^8 conidia $\cdot$ mL^{-1}. For the experiments, spores (10^6 mL^{-1}) were inoculated in 100 mL of liquid Cove's medium (MC) supplemented with 0.05% yeast extract [9]. As a carbon source, crystalline chitin, tick cuticle (*B. microplus*), glucose, or *N*-acetylglucosamine (GIcNAc) was added to the medium at different concentrations. Alanine, glycine, methyonine, and valine (0.5%) were added together with the fungal inoculum and at each 24 h of growth. After 72 h of incubation on a rotating shaking platform (150 rpm) at 27°C, the mycelium was removed by filtration on Whatman No. 1 filter paper. Prior to use in enzymatic assays, the filtrates were dialyzed against 20 mM Tris–HCI buffer (pH 8.0). The total protein content was determined by the Bradford method [6], with a known concentration of BSA as the standard.

Analytical procedure. Subtilisin-like protease was determined with *N*-Suc-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (Sigma). The reaction mixture was 15 μL substrate (2 μM), 10 μL enzyme sample, and 75 μL 50 mM Tris–HCL, pH 8.0. The kinetic assay was done in a Spectra Max 250 and read in a Softmax Pro (405 nm/30 min) (Molecular Devices). Enzyme activity is expressed as nanomoles nitroanilide (NA) released per milliliter per minute at 37°C [22]. The specific activity is represented as units per microgram of protein. Assays were performed in three independent experiments, with four replicates for each sample. Statistical and data analyses were performed using SPSS for Windows (Release 8.0, 1997). Tukey HSD ($p < 0.05$) was used for comparisons of means.

Chitinolytic activity was determined using *N*,*N*′,*N*″,*N*‴-tetracetylchitotetraose (4 mM) to detect endochitinase. The reaction mixture was 40 μL 0.2 mM acetate buffer (pH 5.4)/10 μL substrate/120 μL sample. After 1 h of incubation at 37°C the amount of *N*-acetylglucosamine (GIcNAc) released was determined as described [22]. One unit of chitinase was defined as the amount of enzyme that releases 1 μmol of GIcNAc per minute at 37°C.

Scanning electron microscopy (SEM). Groups of 12 engorged *B. microplus* females were immersed for 30 s in *B. bassiana* or *B. amorpha* conidial suspensions (10^6 conidia $\cdot$ mL^{-1}). Sterile distilled water was applied to the control ticks. After treatment, ticks were maintained in petri dishes at 28°C and 85% relative humidity for up 4 days. For SEM analysis, ticks were fixed overnight at 4°C with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Postfixation was carried out in 1% (w/v) osmium tetroxide in the same buffer. The specimens were rinsed in buffer, dehydrated in a series of 30–100% acetone solutions, dried at critical point in CO_2 (CPD 030 BALTEC), and coated with gold in a sputter-coater (SCD 050 BALTEC). The material was examined in a Jeol JSM 5800 scanning electron microscope at the Centro de Microscopia Electrônica da Universidade Federal do Rio Grande do SuI (CME/UFRGS, Porto Alegre/RS).

Results and Discussion

Fungal extracellular hydrolytic enzymes are important for degradation of the host cuticle during infection, facilitating penetration and providing nutrients for further growth. Like most fungal pathogens, *B. amorpha* and *B. bassiana* might use a combination of enzymes and mechanical force to penetrate the host cuticle and access the nutrient-rich host hemolymph. The effects of different carbon sources on chitinase and protease secretion by *B. amorpha* and *B. bassiana* were tested in medium supplemented with simple or complex carbon sources individually or in combination. As shown in Tables 1 and 2, *B. amorpha* and *B. bassiana* produced chitinases and proteases in all media tested; however, the amount of secreted enzymes varied. The highest levels of endochitinase activity were found in culture supernatants from tick cuticle and chitin for both fungi. Glucose (1%) and GIcNAc (1%) repressed enzyme secretion (Table 1). The effect of glucose repression was previously described for proteins utilized in carbohydrate degradation pathways. According to St Leger et al. [25–27] GIcNAc might cause catabolite repression of chitinases when in excess of the immediate growth requirements of the organisms. In *M. anisopliae*, GIcNAc shows a special dual regulation on chitinase production. It induced the production and secretion of the

Table 1. Effect of carbon sources and amino acids on secretion of chitinases from *B. amorpha* and *B. bassiana*

Substrate	Chitinolytic activity, U (μg protein)/30 min	
	B. amorpha (B 95)	*B. bassiana* (CG 166)
Glucose (1%)	0.368^{g}	0.407^{g}
GlcNAc[a] (1%)	0.006^{l}	0.069^{i}
GlcNAc (0.5%)	0.700^{e}	0.581^{f}
Alanine (0.5%)	0.508^{fg}	0.495^{fg}
Glycine (0.5%)	0.590^{f}	0.521^{fg}
Methionine (0.5%)	1.284^{c}	1.049^{d}
Valine (0.5%)	0.947^{d}	0.957^{d}
Chitin[b] (1%)	2.134^{a}	2.312^{a}
Chitin (0.5%) + glucose (0.5%)	0.439^{fg}	0.485^{fg}
Chitin (0.5%) + GlcNAc (0.5%)	0.290^{h}	0.278^{h}
Chitin (0.5%) + alanine (0.5%)	0.274^{h}	0.279^{h}
Chitin (0.5%) + glycine (0.5%)	0.938^{d}	1.039^{d}
Chitin (0.5%) + methionine (0.5%)	1.997^{a}	1.841^{ab}
Chitin (0.5%) + valine (0.5%)	1.482^{c}	1.731^{ab}
Cuticle[c] (1%)	2.148^{a}	1.978^{a}
Cuticle (0.5%) + glucose (0.5%)	0.414^{g}	0.437^{g}
Cuticle (0.5%) + GlcNAc (0.5%)	0.289^{h}	0.373^{g}
Cuticle (0.5%) + alanine (0.5%)	0.518^{fg}	0.776^{e}
Cuticle (0.5%) + glycine (0.5%)	0.871^{d}	1.015^{d}
Cuticle (0.5%) + methionine (0.5%)	0.573^{f}	0.691^{e}
Cuticle (0.5%) + valine (0.5%)	0.714^{e}	0.903^{de}

Note. The results are means of three independent experiments, with four replicates for each enzymatic assay. Chitinase activity was determined with *N,N′,N″,N‴*-tetracetylchitotetraose. Means followed by the same superscript letter in each column are not significantly different according to Tukey HSD ($p < 0.05$).
[a]*N*-Acetylglucosamine.
[b]Crystalline chitin.
[c]*B. microplus* cuticle.

Table 2. Effect of carbon sources and amino acids on secretion of subtilisin-like protease from *B. amoropha* and *B. bassiana*

Substrate	Proteolytic activity, U (μg protein)/30 min	
	B. amorpha (B 95)	*B. bassiana* (CG 166)
Glucose (1%)	0.1128^{f}	0.1141^{f}
GlcNAc[a]	0.4388^{e}	0.7885^{de}
GlcNAc (0.5%)	0.5706^{e}	0.9095^{de}
Alanine (0.5%)	0.1579^{f}	0.0846^{f}
Glycine (0.5%)	0.6330^{e}	0.0530^{f}
Methionine (0.5%)	0.9767^{de}	1.3245^{e}
Valine (0.5%)	0.7275^{de}	0.9381^{e}
Chitin[b] (1%)	0.0437^{f}	0.0448^{f}
Chitin (0.5%) + glucose (0.5%)	0.0082^{f}	0.0240^{f}
Chitin (0.5%) + GlcNAc (0.5%)	0.0363^{f}	0.0595^{f}
Chitin (0.5%) + alanine (0.5%)	0.0387^{f}	0.0479^{f}
Chitin (0.5%) + glycine (0.5%)	0.0329^{f}	0.0465^{f}
Chitin (0.5%) + methionine (0.5%)	0.0238^{f}	0.0423^{f}
Chitin (0.5%) + valine (0.5%)	0.0711^{f}	0.0563^{f}
Cuticle[c] (1%)	14.1771^{a}	14.3309^{a}
Cuticle (0.5%) + glucose (0.5%)	0.2391^{e}	0.2421^{e}
Cuticle (0.5%) + GlcNAc (0.5%)	1.1617^{d}	1.0655^{d}
Cuticle (0.5%) + alanine (0.5%)	6.2415^{b}	2.5076^{c}
Cuticle (0 5%) + glycine (0.5%)	8.4731^{b}	7.6695^{b}
Cuticle (0.5%) + methionine (0.5%)	3.6235^{c}	3.7963^{c}
Cuticle (0.5%) + valine (0.5%)	1.4435^{d}	2.8830^{c}

Note. The results are means of three independent experiments, with four replicates for each enzymatic assay. Subtilisin-like protease was determined with *N*-Suc-$(Ala)_2$-Pro-Phe-*p*-nitroanilide. Means followed by the same superscript letter in each column are not significantly different according to Tukey HSD ($p < 0.05$).
[a]*N*-Acetylglucosamine.
[b]Crystalline chitin.
[c]*B. microplus* cuticle.

enzyme at low concentrations but repressed chitinase secretion at higher concentrations [3]. This effect was also observed for the extracellular endochitinase of *B. amorpha* and *B. bassiana*. When GIcNAc was added to media at a concentration of 0.5%, chitinase activity was 11- and 8-fold higher compared with 1% GIcNAC for *B. amorpha* and *B. bassiana*, respectively (Table 1). Even when 0.5% GIcNAc was added to a chitin-containing medium, similar results were observed, on a more moderate scale (Table 1).

High levels of subtilisin activity were observed in culture supplemented with tick cuticle for both fungi (Table 2). Since arthropod cuticles comprise about 70% protein, this enzyme may play an important role in host penetration. The addition of alanine (0.5%) to the culture medium repressed subtilisin secretion (Table 2). St Leger et al. [28] verified that in *M. anisopliae*, alanine addition repressed both apressorium formation and the release of subtilisin-like proteases. Alanine is the major amino acid found in the cuticle of insects and also in *B. microplus* [16]. Moreover, comparison between *pr1* cDNA cloned from *B. bassiana* [18] and *pr1* cDNA cloned from *M. anisopliae* [28] showed significant similarity.

Entomopathogenic fungi evolved distinct strategies for their attachment to hosts, varying considerably in their modes of action, virulence, and degree of host specificity [8]. Direct penetration of intact cuticle is the normal mode of entry by most entomophatogenic fungi. *B. amorpha* and *B. bassiana* are not exceptions, and the conidia are capable of germination on the host surface and often differentiate to form apressoria. *Beauveria* comprises two main insect pathogenic species, *B. bassiana* and *Beauveria brongniartii*, which are mainly parasitic on Lepidoptera and Coleoptera [24]. *Beauveria* species are classified by the shape of their conidia and the placement of conidia on the conidiogeneous apparatus [14]. Traditionally, the main difference among the

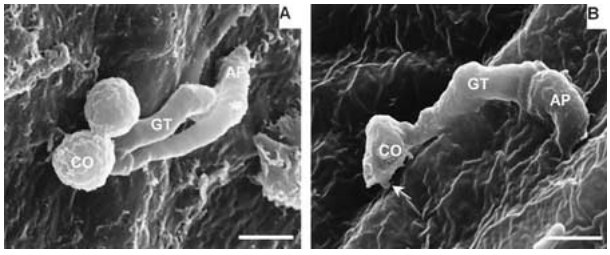

Fig. 1. Scanning electron microscopy of *Booplhilus microplus* cuticle infected by *Beauveria bassiana* (**A**) and *Beauveria amorpha* (**B**). Detail of conidia (CO), germ tube (GT), and appressoria (AP) formation and penetration on the tick epicuticle surface 3 days postinfection. Arrow in B indicates the mucilage layer. Bar = 2 μm.

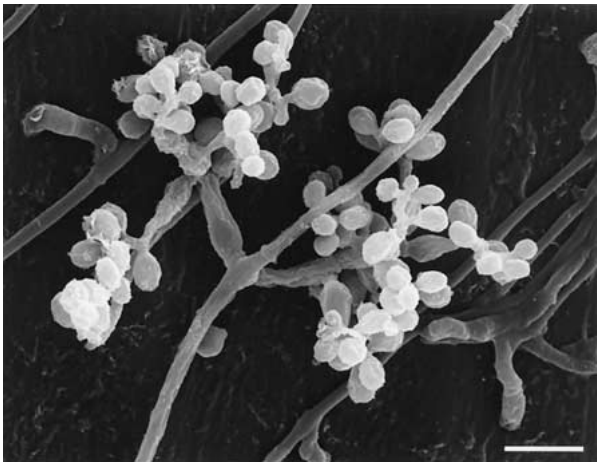

Fig. 2. Detail of *B. bassiana* conidiogenous cells formed in tightly clustered groups. Bar = 5 μm.

most common species is the shape and size of the conidia. SEM analysis of infected *B. microplus* showed that *B. bassiana* and *B. amorpha* conidia are capable of attaching to the epicuticle surface (Fig. 1). The conidial morphology of *B. bassiana* was generally spherical (Fig. 1A), whereas *B. amorpha* conidia on ticks were often flattened on one side as in the original description (Fig. 1B). Adherence of conidia to the host surface is probably mediated by hydrophobic interactions between conidia and the arthropode cuticle [4] and production of an adhesive mucous layer. The fungus produces a thin amorphous mucilage layer and it firmly adheres the conidia and germ tubes to the tick integument (Fig. 1B). The first sign of conidia germination is germ-tube extrusion. Each conidium from both species usually produced only one germ tube that differentiated in apressoria and penetrated the tick cuticle (Figs. 1A and B). *B. bassiana* presented conidiogenous cells formed in tightly clustered groups (Fig. 2), whereas *B. amorpha* sometimes had solitary conidiogenous cells.

For most aspects of *B. microplus* infection by *B. amorpha* and *B. bassiana*, our observations are consistent with the commonly described sequence of events that characterizes other entomopathogenic fungal interactions [2, 8]. The penetration mode of entomopathogenic fungi is similar to that of plant pathogenic fungi and is suggested to be based on a combination of mechanical pressure and enzymatic degradation [28]. Appressoria adhere to the plant surface by secreting a potent glue [11]. The force is exerted vertically and might be efficiently directed to the cuticle [10].

We showed that *B. amorpha* and *B. bassiana* produce subtilisin-like proteases and chitinases in the presence of tick cuticle. The multiplicity of these enzymes provides a major challenge in determining the role played by each particular enzyme in adaptation to a new environment or in pathogenicity. The high capacity of the secretion machinery of these fungi is still to be exploited for biotechnological purposes. However, our knowledge of the fungal secretion pathway is still at an early stage.

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Literature Cited

1. Altshul SF, Gish W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Bio 215:403–410
2. Alves SB (1998) Controle Microbiano de Insetos. São Paulo, Brazil: Fund Est Agrários Luiz de Queiroz, Piracicabat
3. Barreto CC, Staats CC, Schrank A, Vainstein MH (2004) Distribution of chitinases in the entomopathogen *Metarhizium anisopliae* and effect of N-acetyglucosamine in protein secretion. Curr Microbiol 48:102–107
4. Bidochka MJ, St. Leger RJ, Roberts DW (1997) Mechanisms of deuteromycete fungal infections in grasshoppers and locusts: an overview. Mem Entomol Soc Can 171:213–224
5. Bing LA, Lewis LC (1993) Occurrence of entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in diferents tillage regimes and in *Zea mays* L. and virulance towards *Ostrinia nubilalis* (Hübner). Agr Ecos Environ 45:147–156
6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
7. Castrillo LA, Vandenberg JD, Wraigth SP (2003) Strain-specific detection of introduced *Beauveria bassiana* in africultural fields by use of sequence-characterized amplified region markers. J Inv Pathol 82:75–83
8. Clarkson JM, Charnley AK (1996) New insights into mechanisms of fungal pathogenesis in insects. Trends Microbiot 4:197–204
9. Cove DL (1966) The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochiem Biophys Acta 113:51–56
10. Deising HB, Werner S, Wernitz M (2000) The role of fungal appressoria in plant infection. Microbes Infect 2:1631–1641

11. Ebata Y, Yamamoto H, Uchiyama T (1998) Chemical composition of the glue from appressoria of *Magnaporthe grisea*. Biosci Biotech Biochem 62:672–674
12. Frazzon APG, Da Silva Vaz Junior I, Masuda A, Schrank A, Vainstein MH (2000) *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet Parasitol 94:117–125
13. Furlong MJ, Groden E (2003) Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. J Inv Pathol 83:127–138
14. Glare TR, Inwood AJ (1998) Morphological and genetic characterization of *Beauveria* spp. from New Zealand. Mycol Res 102:205–256
15. Goettel MS, Leger RJ, Rizzo NW, Staples RC, Roberts DW (1989) Ultrastructural localization of a cuticle degrading protease prodiced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host cuticle. J Gen Microbiol 135:2223–2239
16. Hackman RH, Goldberg M (1987) Comparative study of some expanding arthropod cuticles: the relation between composition, structure and function. J Insect Physiol 33:39–50
17. Hajek AE, wraight SP, Vandenberg JD (2001) Control of arthropods using pathogenic fungi. In: Pointing SB, Hyde KD (eds). Bioexploitation of filamentous fungi. Fungal. Diversity research series 6. pp 309–347
18. Joshi L, St. Leger RJ, Bidochka MJ (1995) Cloning of cuticle-degrading protease from the entomophatogenic fungus, *Beauveria bassina*. FEMS Microbiol Lett 125:211–218
19. Kaaya GP, Hassan S (2000) Entomogenous fungi as promising biopesticides for tick control. EXP Appl Acarol 24:913–926
20. Kaaya GP, Munyinyi DM (1995) Biocontrol Potential of the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* for tsetse flies (Glossina ssp.) at developmental sites. J Inv Pathol 66:237–241
21. Kaaya GP, Mwangi EM, Ouna EA (1996) Prospects for biological control of livestock tick *Rhipecephalus appendiculatus* and *Amblyomma variegatum*, using the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae*. J Inv pathol 67:15–20
22. Krieger de Moraes C, Schrank A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr Microbial 46:205–210
23. Monteiro AC, Fiorin AC, Correia ACB (1998) Pathogenicity of isolates of *Metarhizium anisopliae* (Metsch.) Sorokin towards the cattle tick *Boophilus microplus* (Can.) (Acari: Ixodidae) under laboratory conditions. Rel Microbiol 29:109–112
24. Muro MA, Mehta S, Moore D (2003) The use of amplified fragment length polymorphism for molecular analysis of *Beauveria bassiana* isolates from Kenya and other countries, and their correlation with host and geographical origin. FEMS Microbiol Lett 229:242–257
25. St Leger RJ, Cooper RM, Charnley AK (1986a) Cuticle-degrading enzymes of entomopathogenic fungi: cuticle degradation in vitro by enzymes from entomopathogens. J Inv Pathol 47:167–177
26. St Leger RJ, Cooper RM, Charnley AK (1986b) Cuticle-degrading enzymes of entomopathogenic fungi: mechanism of interaction between pathogen enzymes and insect cuticle. J Inv Pathol 47:295–302
27. St. Leger RJ, Cooper RM, Charnley AK (1986c) Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture cuticle. J Inv Pathol 48:85–95
28. St Leger RJ, Cooper RM, Charnley AK (1987) Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Arch Biochem Biophys 253:221–232
29. St. Leger RJ, Allee LL, May B, Staples RC, Roberts DW (1992) World-wide distribution of genetic variation among isolates of *Beauveria* spp. Mycol Res 96:1007–1015
30. St Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119–S1125