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Comparative Biochemistry and Physiology, Part C 145 (2007) 333–338
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Screening and secretomic analysis of enthomopatogenic *Beauveria bassiana* isolates in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton

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Received 17 August 2006; received in revised form 22 December 2006; accepted 9 January 2007
Available online 1 February 2007

Abstract

The production of cowpea (*Vigna unguiculata*), an important self-sustained crop in Latin America and Africa, is severely affected by damage by the cowpea weevil *Callosobruchus maculatus.* The presence of a single larva in stored seeds can lead to losses of almost 40%. Control of *C. maculatus* currently relies on the inefficient use of chemical insecticides and post-harvest treatments. The use of entomopathogenic fungus became a reliable alternative for coleopteran pest control and has been extensively investigated. Among them, *Beauveria bassiana* and *Metarhizium anisopliae* were widely evaluated in order to measure their virulence toward many insects. In this report, we evaluated the insecticidal activity of ten strains of *B. bassiana* and the most lethal fungi strains were analyzed for proteinaceous secretions by two dimensional electrophoresis and for enzyme activities, including chitinolytic, proteolytic and α-amylolytic activities. This study could, in the near future, help to establish novel biotechnological tools to use for cowpea weevil control.

Keywords: Chitinase; Proteinase; Secretomic; *Beauveria bassiana*; *Callosobruchus maculatus*; Two-dimensional gel

1. Introduction

Many poor farmers in South America and Africa utilize cowpea seeds (*Vigna unguiculata*) as self-sustained crop (Delincée et al., 1998), which is severely attacked by cowpea weevil *Callosobruchus maculatus*. This bruchid, in larval stage, is able to attack stored grains, penetrating seed and seedpods to feed on cotyledons. This predation significantly reduces the quantity and quality of seeds destined for human consumption and sowing purposes. The development of a single larva in stored grains can lead to severe losses (Credland et al., 1986). Control of *C. maculatus* currently relies on several strategies as the use of chemical insecticides (Tederson et al., 2006), biological control (Jackai and Adalla, 1997) by using bacteria, protozoa, nematodes, virus and fungus (Chapman, 1974; Kaya and Gaugler, 1993; Legner, 1995; Becker and Ascher, 1998; Scholte et al., 2004) and also post harvest treatment by ionizing radiation (Delincée et al., 1998). Furthermore, the use of entomopathogenic fungus became a reliable alternative for coleopteran pests control as an alternative to chemical controls and has been extensively investigated (Adane et al., 1996; Moino et al., 1998; Rice and Cogburn, 1999; Bourassa et al., 2001; Kassa et al., 2002). *Beauveria bassiana* and *Metarhizium anisopliae* were world widely evaluated according to their virulence toward storage maize bruchid pests (Cherrya et al., 2005), showing that their proteinaceous secretions could be utilized as biotechnological tools in the development of novel bioinsecticides and/or construction of resistant genetic modified

Abbreviations: MM, Minimal Medium; MMC, Minimal Medium+0.5% *C. maculatus* exoskeleton; PDA, potato dextrose agar; 3.5 DNS, 3.5 dinitrosalicylic acid; IPG, immobilized pH gradient; DTT, dithiothreitol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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doi:10.1016/j.cbpc.2007.01.010

plants. In a previous study, we screened ten *M. anisopliae* isolates with virulence against *C. maculatus* and also performed enzymatic and proteomic analyses by two dimensional gels of secretion produced in presence of *C. maculatus* shells (Murad et al., 2006). Therefore, the deuteromycete *B. bassiana* is also capable to penetrate through the insect cuticle, secreting hydrolytic enzymes such as chitinases, proteinases and lipases, commonly referred as cuticle-degrading enzymes (Leger et al., 1986; Moraes et al., 2003; Fang et al., 2005) being effective toward several bruchids (Shah and Pell, 2003; Cherrya et al., 2005). In this report, insecticidal activities of ten *B. bassiana* isolates were evaluated and most lethal fungi had their proteinaceous secretion analyzed by two-dimensional electrophoresis as well as by their enzymatic activity which includes chitinolytical, proteolytical and α-amylolytic activities, in order to establish novel biotechnological tools to use in cowpea weevil control. A brief comparison to proteome analyses of *M. anisopliae* secretion was also provided to shed some light over the virulence mechanisms of mycelious entomopathogenic fungi toward insect-pests.

2. Material and methods

2.1. Insects and Isolates of B. bassiana

C. maculatus colonies were maintained in flasks (20 mL) containing cowpea seeds (*V. unguiculata*) and were incubated at 28 °C with 60–70% UR. Two or three days a week, 20–30 couples (24–48 h old) were transferred to new containers with cowpea seeds, for female oviposition. After 48 72 h, insects were removed and the flasks with *C. maculatus* eggs were incubated for larval and pupal development. All adult insects used in bioassays were 24–48 h older. Biochemical assays were carried out using adults removed from colonies 24 h after oviposition. These insects were dehydrated at 49 °C for 48 h and macerated for posterior use in bioassays. Initial screenings were performed using ten isolates of *B. bassiana* obtained from Entomopathogenic Fungal Collection of Embrapa Recursos Geneticos e Biotecnologia (Brasília, Brazil), preserved in liquid nitrogen. Most of isolates used in these work were obtained from colonized coleopteran insect–pests (Table 1).

Table 1
B. bassiana isolates screened against *C. maculatus*

Isolate Number*	Host	Local	Concentration (conida ml^{-1})
CG02	*Elaeidobius sp*	AM-Brazil	1.04×10^8
CG05	*Coleoptera*	DF-Brazil	1.00×10^8
CG07	*Leptinotarsa decemlineata*	U.S.A.	1.25×10^8
CG08	*Coleoptera*	DF-Brazil	1.00×10^8
CG11	*Nezara viridula*	PR-Brazil	1.25×10^8
CG12	*Cycloneda sanguinea*	PR-Brazil	1.26×10^8
CG15	*Lebia concinna*	PR-Brazil	2.20×10^8
CG17	*Hypothenemus hampei*	SP-Brazil	1.00×10^8
CG26	*Coleoptera*	DF-Brazil	1.53×10^8
CG52	*Coleoptera*	DF-Brazil	1.02×10^8

2.2. Isolates screening and pathogenicity studies

Fungi utilized in bioassays were grown in PDA (potato dextrose agar) medium for 15 days at 28 °C. For an initial screening, conidial were suspended in 0.1% Tween 80 to obtain a dilution of 1 to 4×10^8 conidial mL^{-1} (Table 1). Conidial solutions (2.0 mL) were sprayed upon insects using a Potter Spray Tower (Burkard Manufacturing, Hertfordshire, England). Negative control treatment was done spraying 2.0 mL of 0.1% Tween 80 solution. Before application, adult insects were immobilized using a CO_2 flux. Three replicates (ten insects each) were performed for each isolate. After applications, insects were conditioned in flask containers (10 mL) with 20 cowpea seeds an incubator chamber at 28 °C and observed daily for mortality assessment. Dead insects were maintained in humidified chambers to confirm if mortality was caused for fungal infection. For bioassays, conidial suspensions, at 5 different concentrations, were used (CG5: 1.41×10^5, 10^6, 10^7, 10^8 and 10^9 conidial mL^{-1}; CG7: 1.61×10^5, 10^6, 10^7, 10^8 and 10^9 conidial mL^{-1}), using 0.1% of Tween 80 solution as negative control. Each isolates dosage was measured in triplicate ($n=10$ insects per replicate). Reference values (Vicentini et al., 2001) were used to calculate the correct number of conidial deposited per unit of area in each fungal application procedure. After that, mortality evaluation was done in the same way used in screening bioassays. CL50, regression parameters and significance of each isolate were calculated using Probit method by software Micro Probit 3.0 (Thomas and Sparks, 2001). Superpositions of confidence intervals of 95% were use to test statistical differences in CL50's of isolates. Furthermore, *t*-test was used to compare slope of regression equation.

For further analyses, *B. bassiana* CG05 isolated from *C. maculatus* exoskeleton was grown in a complete medium (0.0001% $FeSO_4$, 0.05% KCl, 0.15% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.6% $NaNO_3$, 0.0001% $ZnSO_4$, 0.15% casein hydrolyzed, 0.05% yeast extract, 1% glucose, 0.2% peptone and 2% agar) being incubated at 28 °C for 10 days. In order to carried gels and enzymatic analysis, 10^7 spores were inoculated in TM medium containing 0.1% bactopeptone, 0.03% Urea, 0.2% of KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.03% $MgSO_4$ ($7{\cdot}H_2O$), 0.3% $C_6H_{12}O_6$ and $FeCl_3$ as trace element. After three days of incubation, the mycelia was transferred to minimal medium (MM) (0.2% KH_2PO_4, 0.03% $MgSO_4$, 1.4% $(NH_4)_2SO_4$ and $FeCl_3$ as trace element, and to MM containing 0.5% *C. maculatus* dehydrated cuticle (MMC). Both were re-incubated for three days at 29 °C in a rotation of 130 rpm. Both media were double filtered and stored at −20 °C for further analysis.

2.3. Protein quantification

Bradford method (Bradford, 1976) was used for protein quantification, where 1.0 mL of Bradford reagent (100 mg of Coomassie brilliant blue, 50 mL of ethanol 95%, 100 mL of phosphoric acid 85%) was added to sample (100 μL). Distilled

water was used as negative control. Optical densities were measured by spectrophotometer at 595 nm and values were converted to protein concentration (μg mL^{-1}) using bovine serum albumin (BSA) standards.

2.4. Serine proteinase assays

Proteolytical activity from fungi secretion was evaluated according to Leger et al. (1987), were 100 μg of secreted proteins were added in tubes containing 500 μL of 5% casein pH 8.0, which was used as substrate. 100 μL of 0.05 M Tris–HCl buffer, pH 8.5 was added to reaction. After incubation for 30 min at 40 °C, 1500 μL of 5% trichloroacetic acid (TCA) was added to stop enzymatic reaction. A negative control was done inactivating enzyme adding TCA before incubating. Samples were centrifuged at 10,000 *g* for 15 min at 4 °C and supernatant optical densities were measured at 280 nm. Each assay was carried out in triplicate.

2.5. Chitinolytic assays

Aiming to identify enzymes capable to hydrolyze chitin, assays were done at a standard concentration of 164 μg mL^{-1} as observed in protein quantification by Bradford method (1976). For negative control, 500 μL of MMC and MM in 0.05 M sodium acetate buffer pH 5.0 were boiled and added in flasks containing 500 μL of 1% colloidal chitin in the same buffer prepared according Aronson et al. (1967). The same was done with MMC and MM non-boiled samples. Colloidal chitin was prepared using 20 g of chitin from crab shells (Sigma). This compound was eluted in 500 mL of 12 M HCl under agitation for 4 h at 4 °C. The solution was filtered and washed with 500 mL of 50% ethanol until white powders begin to precipitate. 200 mL of deionized water was added to solution and further precipitated for 15 h at 4 °C. Pellet was separated and washed several times with MiliQ water, until the pH reached 7.0. The final solution was lyophilized and stored at room temperature. Chitinolytic assays, carried out in triplicate, were incubated for 6 h at 37 °C. Samples (250 μL) were transferred to tubes containing 1 mL of 1% 3.5 dinitrosalicylic acid (3.5 DNS) and boiled for 5 min. Both reactions were analyzed by spectrophotometer at 530 nm. An *N*-acetyl-D-glucosamine standard curve was used to achieve the reducing sugar concentration as described by Miller (1959).

2.6. α-Amylolytic activity

α-Amylase activity was determined according method described by Bernfeld (1956) in 250 μl of sodium acetate buffer 0.05 M, pH 6.8. 1% starch was added as substrate to reaction and each fraction was incubated at 37 °C for 20 min. A negative control was done boiling samples before incubation. Enzymes activities were determined by adding 1.0 mL of 3.5 DNS (1% dinitrosalicylic acid, 0.2 N NaOH, 30% Na–K-potassium tartrate) and absorbance were measured at 530 nm. Each assay was carried out in triplicate.

2.7. Gel electrophoresis analysis

The isoelectric focusing and molecular mass separation were done using the method described by Gorg et al. (1988) on 13 cm immobilized pH gradient (IPG) strips with pH range of 3–11 and a Multiphor II electrophoresis system from General Electric. 200 μg of MMC and MM were added in 1.5 mL microtube and were precipitated using 2D Clean - Up Kit (GE HealthCare) and than resuspended in a 250 μL of solution containing 2% CHAPS, 8M urea, 7 mg dithiothreitol (DTT) and 2% IPG Buffer. Strips were hydrated with solution described above for 16 h. Isoelectric focusing was carried out for 30 min in 500 V, 30 min 1000 V and 380 min in 3500 V, 2 mA and 5 W. After first dimension, strips were equilibrated in a solution containing 6 M urea, 1% DTT and 2% SDS for 15 min and then applied in gels. Second dimension were performed in 18×24 cm SDS-PAGE 12.5% gels as described by Laemmli (1970), as well SDS - PAGE minigel 12.5%. In both, bromophenol blue was used as tracking dye. Electrophoreses were done at 250 V, 80 mA and 10 W for 7 h. Gels were silver stained.

3. Results and discussion

To improve the knowledge of entomopathogenic fungi secretome in response to bean bruchids, *B. bassiana* isolates were evaluated. Comparing results here presented with our previous work (Murad et al., 2006), *B. bassiana* screening strains showed that this fungus is more virulent than to *M. anisopliae*. All *B. bassiana* isolates tested caused mortality to *C. maculatus*. Therefore only two isolates (CG05 and CG07) demonstrated mortality higher than 50% (Table 2) and only one isolate show significantly different mean mortality value. Pathogenicity values showed no statistical differences in CL50 between both isolates (confidence intervals superposition criteria $p<0.05$). The CL50 of tested isolates were in the range of 1 to 5×10^4 conidia cm^{-2} (Table 3). In the same way, slope of regression equation not show statistical differences indicating that the isolates tested have the same dynamic pathogenicity,

Table 2
Mortality (Media values±SE) of *C. maculatus* caused by different *B. bassiana* isolates

Isolate	Mortality (%)
CG05	66.3 (±28.47) a
CG07	54.5 (±9.79) ab
CG26	39.17 (±11.76) ab
CG11	25.9 (±9.02) ab
CG15	19.4 (±10.3) ab
CG17	16.67 (±16.67) ab
CG12	9.1 (±5.25) ab
CG8	6.37 (±3.19) ab
CG2	5.1 (±2.95) ab
CG52	3.33 (±3.33) b
Control	0

Values followed by the same minus letter do not show statistical differences (ANOVA $F_{9,20}$: 3,25 $P=0.013$, SNK test $P>0.05$). Data of control treatment were not included in the analyses.

Table 3
CL50 (95 % fiducial limits) and regression parameters *a* (*y*-intercept) and *b* (slope) (estimated by probit method) for two fungal isolates tested in adults of *Callosobruchus maculatus*

B. bassiana	CL 50(conidia cm^{-2})	*a*	b±SE	χ^2
CG05	$2.7\times10^4(1.45\times10^4-4.89\times10^4)$	−1.09	1.37±0.22 a	1.28 ns
CG07	$5.05\times10^4(2.83\times10^4-8.56\times10^4)$	−1.39	1.35±0.21 a	3.96 ns

χ^2 indicate the adjustment of data to probit model.
Reference: Values of *b* followed by the same letter do not show statistical differences.
$t=0.0658$ $df=8$, $P=0.949$.

especially when related to concentration. These are surprising results, once that the isolates utilized in this report demonstrated a shorter germination time, as previously observed in *M. anisopliae* (Murad et al., 2006). The latter shows a rapid growth and an enhanced spore production, conferring beneficial characters to *M. anisopliae* for biofactory applications. Nevertheless *B. bassiana* is a pathogen much more aggressive and should also be considered.

As formally described, supernatant obtained from minimal media in the presence (MMC) and absence (MM) of *C. maculatus* shells were quantified and enzymatic assays were also performed, using the most virulent isolate CG05. After 3 days of incubation time, 164 μg mL^{-1} protein concentration was observed in MMC and only 33 μg mL^{-1} in MM indicating a clear raise in protein secretion induced by *C. maculatus* exoskeleton. Moreover, enzymatic assays confirmed a higher proteolytic and chitinolytic activity under insect shells induced broth (Fig. 1A,B). This data are in agreement to observe in grasshopper *Melanoplus sanguinipes* cuticle inoculated with the same fungi (Bidochka and Khachatourians, 1994). An α-amylolytic assay was also performed, but no activity was detected (Fig. 1C). Similar data was also observed in secretion obtained from *M. anisopliae* (Murad et al., 2006). This was also expected since insect shells do not have starch in their composition.

The samples were further analyzed by a 12.5% SDS-PAGE. 100 μg of each sample were applied in the gel (Fig. 2) showing proteins produced in MM and MMC. A wide range of proteins molecular masses could be visualized in MMC being more expressive bands indicated by arrows with 28 kDa, 33 kDa, 55 kDa and 120 kDa (Fig. 2, Lane III). On the other hand, MM produced a less expressive range of proteins with approximate masses of 22 kDa and 55 kDa (Fig. 2, Lane II). *M. anisopliae* produced a different protein expression pattern, showing proteins with molecular masses of 27 kDa, 30 kDa, 37 kDa, 45 kDa, 50 kDa and 80 kDa bands (Murad et al., 2006). For a more accurate analysis, samples were analyzed in 2D gels, showing a remarkable difference in protein expression between MMC and MM (Fig. 3). Only 4 spots can be verified in MM (Fig. 3A), while 13 significant spots were identified in MMC (Fig. 3B). All spots from MM gel could be found at the same positions in MMC with molecular masses of 10 kDa and 48 kDa (Fig. 3 — circles). All other spots verified in MMC were induced by the presence of insect shells with expressive spots at 27 kDa and between 40 kDa and 50 kDa (Fig. 3 — diamonds and squares).

Compared to *M. anisopliae* secreted proteins (Murad et al., 2006), *B. bassiana* CG05 express proteins with a higher wide variability of molecular mass and pI range. This could be an important point to determine the higher virulence found in pathogenicity assays. One of them is the production of toxins as beuvericin, a cyclic hexadepsipeptide secondary metabolite that compromise the immunologic system of several arthropods as *Artemia salina* (Calo et al., 2003). Furthermore, some *B. bassiana* isolates also produces melanin, a toxic factor that causes high lethality for lepidopteran *Galleria mellonella*

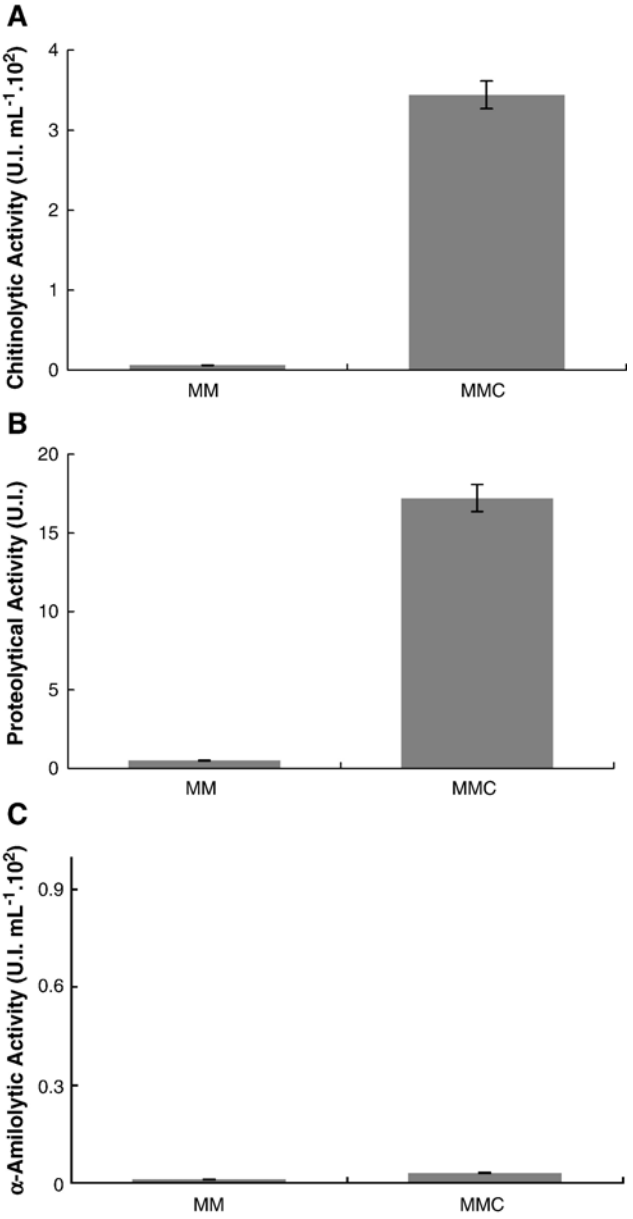

Fig. 1. Comparative chitinolytical (A), proteolytical (B) and α-amylolytic (C) activities of *B. bassiana* secretion in the absence (MM) and presence (MMC) of *C. maculatus* exoskeleton. Vertical bars correspond to standard deviation. Each assay was carried out in triplicate. For chitinolytic and α-amylolytic activity, U.I. indicates enzyme units necessary to produce 1μmol of *N*-Acetyl-D-glucosamine and glucose per minute respectively. For proteolytic, U.I. indicates enzyme units necessary to change 0.1 in O.D. at 280 nm.

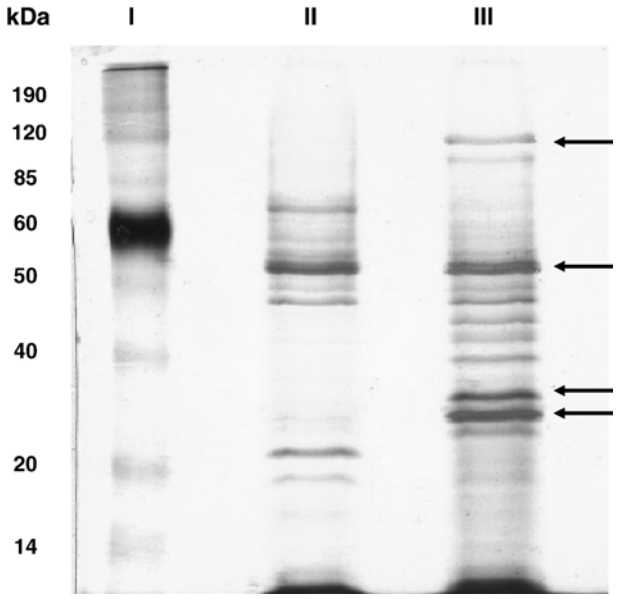

Fig. 2. SDS-PAGE analysis of *B. bassiana* secreted proteins in the absence (Lane II) and presence (Lane III) of 0.5% of *C. maculatus* shells. Lane I corresponds to molecular weight marker. Dark arrows correspond to more expressive bands.

(Fuguet et al., 2004). These two compounds have very low molecular masses and may not be visualized in 2D gels, in despite of their importance in pathogenicity. Chitinolytic enzymes with 45 kDa and 110 kDa were purified from *B. bassiana* isolates that have being growth in colloidal chitin (Havukkala et al., 1993). Furthermore, a 33 kDa (pI 5.4) chitinase was also cloned by Fang et al. (2005) in this same fungus. Therefore, Fuguet et al. (2004) also purified a thermolabile chitosanase-like protein (Bclp) with molecular mass of 28 kDa and pI 4.0, which have cytotoxic effects on *G. mellonella.* This enzyme causes strong cellular alterations in hypodermis, tracheae, and blood cells of larvae, playing an important role in entomopathogenesis. According these data we marked with diamonds the probable positions of these three proteins in the 2D gel (Fig. 3B) with numbers 3, 4 and 5 respectively. Furthermore, endo-proteinases have also being found in *B. bassiana* isolates. One isoform showed molecular mass of 26 kDa and predicted pI between 8.8–10 (Joshi et al., 1995). In addition, a variant proteolytic enzyme found by Urtz and Rice (2000) showed a lower isoelectric point (pI 7.5) and was 0.5 kDa smaller. These spots could be also observed in the gel (Fig. 3B) and were square marked in positions 1 and 2 respectively. In conclusion, our analysis suggested that CG05 might produce similar proteinases and chitinases described, once they have been supported by enzymatic assays (Fig 2). An answer for other spots detected in gel may come from hydrolysis of *C. maculatus* cuticle and other enzymes and proteins no identified until now. Results here reported shed some light over the mechanisms of insect colonization by entomopathogenic fungi. Some previous reports described the proteomic analyses of mycelial fungi. Bidochka and Khachatourians (1994) carry out a proteomic approach in cuticle from grasshopper *Melanoplus sanguinipes* treated with extracellular proteases from entomopathogenic fungi showing 200 different proteins with masses varying between 30 kDa and 90 kDa in untreated cuticle and about 30 spots in treated cuticle with the same mass range. Other fungi proteomes shows a wide range of proteins in different situations. Carberry et al. (2006) analyzed major intracellular proteins from *Aspergillus fumigatus*, showing around 250 spots in which 44% of the proteins were involved in energy metabolism. Finally, hydrolytic enzymes in mycelia and secretion proteins were detected by proteomic

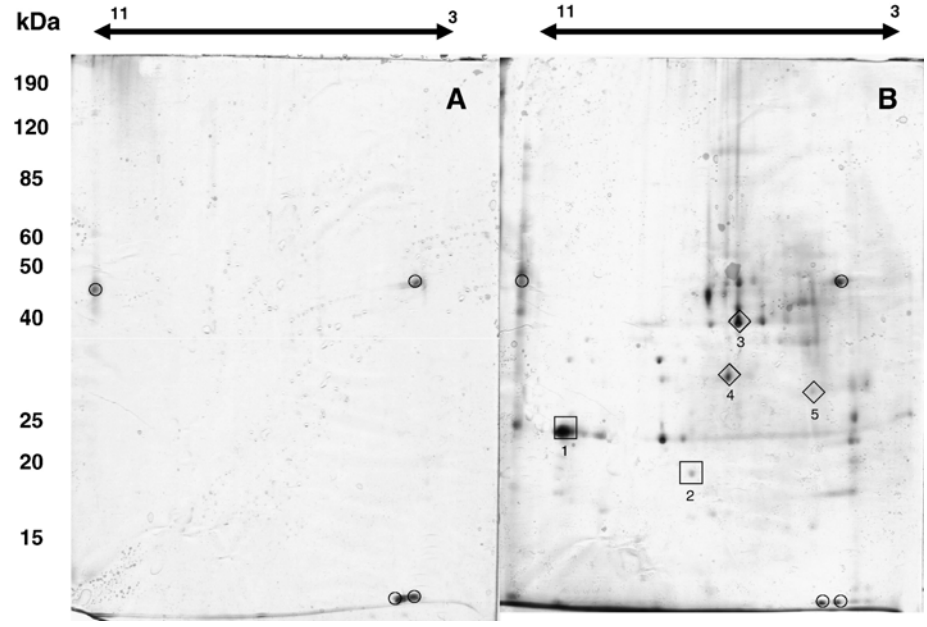

Fig. 3. MM (A) and MMC (B) samples analyzed by two-dimensional gels. Circled spots identify identical matches in MMC and MM gels. Putative enzymes located at positions 1 and 2 (square marked), 3, 4 and 5 (diamond marked) are 26.0 kDa and 21.0 kDa endoproteases, 45.0 kDa and 33.0 kDa chitinases and 28.0 kDa chitosanase-like protein respectively.

approach in fungi biocontrol response of *Trichoderma atroviride* (Grinyer et al., 2005). To elucidate function and confirm their rules in fungal colonization, further studies will be carried out using peptide mass finger printing and Edman degradation sequencing. These data may be used in the development and production of novel specific bioinsecticides, non-target organism safe, and also in engineering of transgenic plants for enhancing the bruchid resistance.

Acknowledgments

UCB, CAPES and CNPq supported this work. We also thank Krishna B. Bonavides for her technical assistance in enzyme assays.

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