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Enzymatic activities and effects of mycovirus infection on the virulence of *Metarhizium anisopliae* in *Rhipicephalus microplus*

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ABSTRACT

The present study aimed to evaluate the pathogenic potential of different *Metarhizium anisopliae* s.l. isolates and to determine whether differences in enzymatic activities of proteases, lipases and chitinases and infection with mycoviruses affect the control of *Rhipicephalus microplus* achieved by these fungal isolates. Engorged female ticks were exposed to fungal suspensions. The lipolytic and proteolytic activities in the isolates were evaluated using chromogenic substrates and the chitinolytic activity was determined using fluorescent substrates. A gel zymography was performed to determine the approximate size of serine proteases released by *M. anisopliae* isolates. To detect mycoviral infections, dsRNA was digested using both RNAse A and S1 endonuclease; samples were analyzed on an agarose gel. Four of the five isolates tested were infected with mycovirus; however, the level of control of *R. microplus* ticks achieved with the only isolate free of infection (isolate CG 347) was low. This finding suggests that mycoviral infection does not affect the virulence of fungi against ticks. Although all five isolates were considered pathogenic to *R. microplus*, the best tick control and the highest levels of enzymatic activity were achieved with the isolates CG 629 and CG 148. The *in vitro* activities of lipases, proteases and chitinases produced by *M. anisopliae* s.l. differed among isolates and may be related to their virulence.

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1. Introduction

Rhipicephalus microplus is one of the main ectoparasites of cattle in tropical and subtropical regions around the world (Powell and Reid, 1982); this organism causes decreases in productivity (Jonsson, 2006) and consequent economic losses to producers (Grisi et al., 2002). Moreover, this parasite is a vector of pathogenic agents that cause bovine diseases with high morbidity (Jonsson et al., 2008).

The use of acaripathogenic (AP) fungi to control ticks has been studied for at least two decades (Bittencourt et al., 1992). Among the fungi studied, *Metarhizium anisopliae* sensu lato (s.l.) is the species that has shown the greatest potential to infect various species of ticks (Fernandes and Bittencourt, 2008). The use of these microorganisms is suitable for the control of arthropods because they are

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able to invade the cuticle of their host (Alves, 1998). The cuticle penetration process starts with conidial adhesion and is followed by germination, modification and specialization of these spores into germ tubes that release enzymes (lipases, proteases and chitinases, among others) to hydrolyze components of the cuticle; concomitantly, germ tubes also exert physical pressure on the cuticle of the arthropods, thus allowing the fungus to invade the hemocoel (Bittencourt et al., 1999; Arruda et al., 2005).

To determine the importance of enzymes that are secreted during fungal infection, studies have been conducted to describe the interactions that take place between a fungus and its arthropod host (Schrank and Vainstein, 2010). Pr 1 subtilisin-like proteases were the first proteases involved in the fungal penetration process to be described (St Leger et al., 1996); the role of chitinases in this process was subsequently described (Silva et al., 2005), and, more recently, Beys da Silva et al. (2010a) demonstrated the importance of lipases in AP fungal infections.

Currently, thousands of *M. anisopliae* isolates from different hosts and substrates are stored in collections around the world. The virulence of these isolates in ticks varies greatly (Quinelato et al., 2012). Studies have also shown that enzymatic secretions, which may influence the virulence of these organisms, vary among different *M. anisopliae* isolates (Mustafa and Kaur, 2009). Double-stranded RNA (dsRNA) viral infections, which can reduce the infective capacity of the fungus, have also been found to influence the virulence of phytopathogenic and acaripathogenic fungi (McCabe et al., 1999). These mycoviruses have been found to infect several fungal genera (Frazzon et al., 2000). The majority of mycovirus infections are latent; however, in some cases, the presence of these organisms can interfere with the fungal phenotype by changing the morphology of the colony and fungal growth and sporulation rates (Melzer and Bidochka, 1998; Chu et al., 2002; Tsai et al., 2004). In the case of the *Metarhizium* genus, the association between viral infections and the virulence of the fungus remains unclear, as studies have shown that dsRNA-infected fungal isolates can be more or less virulent in arthropods than non-infected fungal isolates (Frazzon et al., 2002; Martins et al., 1999).

Accordingly, the present study aimed to evaluate the pathogenic potential of different *M. anisopliae* s.l. isolates and to determine whether differences in enzymatic activities of proteases, lipases and chitinases and infection with mycoviruses affect the control of *R. microplus* achieved by these fungal isolates.

2. Materials and methods

2.1. Obtention of M. anisopliae isolates and maintenance of fungal colonies

Five *M. anisopliae* s.l. isolates were used in the present study: CG 32, *Mahanarva* posticata (Homoptera: Cercopidae), Bahia, 1984; CG 112, *Deois flavapicta* (Homoptera: Cercopidae), Distrito Federal, 1988; CG 148, *Deois flavapicta*, Mato Grosso do Sul, 1982; CG 347, Soil, Goiás, 1991; and CG 629, *Mahanarva posticata*, Alagoas, 1997. The isolates were obtained from Empresa Brasileira de Pesquisa Agropecuaria, Brasília, Brazil. Isolates were chosen based on a previous study (Quinelato et al., 2012; Perinotto et al., 2013) that demonstrated enormous variation in the virulence of these organisms in *R. microplus* larvae and adults respectively. From this previous study, five isolates were selected: the two most virulent isolates, the two least virulent isolates and the isolate with intermediate virulence. The isolates were cultured on 23 ml of potato dextrose agar medium (PDA) (Himedia, Mumbai, India) at 25 ± 1 °C and ≥80% relative humidity (RH) for 15 days.

2.2. Fungal suspensions

Conidia of each isolate were suspended in sterile distillated water with 0.01% Tween 80 (Sigma) (Luz et al., 1998) and quantified according to the method described by Alves (1998).

2.3. Conidial viability

Conidia viability was determined by plating an aliquot (~50 μL) of conidial suspensions on PDA and incubating the sample at 25 ± 1 °C and ≥80% RH for 24 h. Conidial germination was calculated with the method described by Alves (1998).

2.4. Bioassay with engorged tick females

Engorged females were weighed and distributed into six groups with 10 females in each group. They were then individually immersed in fungal suspensions or in the control solution (sterile distillated water plus 0.01% Tween 80) for three minutes. Ticks were fixed on Petri plates and incubated at 27 °C and ≥80% RH. The following biological parameters were investigated: egg mass weight (EMW) evaluated daily, larval hatching percent (LHP) evaluated at tenth day after the treatment, egg production index (EPI) and nutrient index (NI) according to the methods described by Bennett (1974). The percent control (CP) achieved with different *M. anisopliae* s.l. isolates was calculated according to the method of Drummond et al. (1971).

2.5. Fungal re-isolation

Female ticks from non-treated and treated groups were incubated in moisture chambers at 27 ± 1 °C and ≥80% RH for further confirmation of fungal characteristics Rombach et al. (1986).

2.6. Cultivation of isolates for enzymatic assays

One mL of each suspension (10^6 conidia mL^{-1}) was cultivated in an Erlenmeyer flask with 50 mL of minimal liquid medium (Beys da Silva et al., 2010b) supplemented with 1% *R. microplus* cuticle and 1% cholesterol stearate (Sigma Chem., Co., St. Louis, USA). Culture flasks were incubated for 24 h, 48 h or 72 h in an orbital shaker operating at 150 rpm at 25 °C. The extraction of enzymes was performed by adding10% Triton X-100 to the flasks (Silva et al., 2005). Mycelia were then filtered and the supernatant was recovered and stored at −80 °C.

2.7. Enzymatic assays

2.7.1. Lipolytic activity

The lipase activity was determined by analyzing the hydrolysis of the chromogenic emulsion substrate ρ-nitrophenyl palmitate (ρNPP – Sigma) produced with a 1:9 dilution of A solution [ρNPP dissolved in isopropanol (1:3, m/v)] and B solution (450 mL 50 mM Tris–HCl pH 8.0/2 g Triton X-100/500 mg Arabic gum] (Beys da Silva et al., 2010b). The assay was performed in a 96-well plate. Samples of the supernatant of the liquid medium fungal cultures (10 μL–0.16 μg total protein) were mixed with 90 μL of substrate solution; the absorbance was spectrophotometrically measured at 410 nm using a plate spectrophotometer Spectramax (Molecular Devices, Sunnyvale, USA). The lipolytic activity was calculated according to the method described by Beys da Silva et al. (2010b).

2.7.2. Proteolytic activity

The serine protease activity was determined by evaluating the hydrolysis of azocasein (Sigma). Each assay was performed using 100 μL of azocasein 2%, 100 μL of sample (∼1.6 μg total proteins), and 200 μL of 0.05 M sodium phosphate pH 7.9 (Sangorrín et al., 2001). Assays were incubated at 50 °C for 15 min and the reactions were interrupted by the addition of 800 μL of 20% trichloroacetic acid (Merck). Tubes were centrifuged at 4000 × g for five minutes and the absorbance was measured at 400 nm using a spectrophotometer. Enzymatic activity was calculated according to the method of Sangorrín et al. (2001).

2.7.3. Chitinolytic activity

Chitinolytic activity was determined by analyzing the hydrolysis of methylumbelliferyl-triacetylchitotriose (Sigma). Each sample [20 μL (∼0.32 μg total protein)] was added to 5 μL of 0.08 mM substrate and 80 μL of 0.1 M phosphate citrate buffer (pH 5.0). The assays were incubated for 30 min at 37 °C. The reaction was interrupted by the addition of 120 μL of 0.1 M glycine/NaOH buffer (pH 10.6) followed by a 5 min incubation at 37 °C. The absorbance was measured in a fluorimeter (Spectramax) with excitation at 355 nm and emission at 469 nm. Enzymatic activity was measured based on a methylumbelliferone curve (Santi et al., 2010a).

2.8. Total protein amounts

Protein amounts were quantified according to the method described by Bradford (1976) using bovine serum albumin as the standard.

2.9. In gel zymography of proteases

The approximate size of proteins released in the extracellular liquid medium of *M. anisopliae* s.l. after 72 h of growth was measured. Twelve percent sodium dodecyl sulfate (SDS) polyacrylamide gels containing 0.1% ultrapure gelatin (Sigma) were used in the electrophoresis. Fifty micrograms of total protein were applied in each well; the "Prestained SDS-PAGE Standards" (Bio-Rad) molecular weight size markers were used. After electrophoresis, gels were incubated in 50 mM Tris–HCl buffer pH 8.0 with Triton X-100 (Merck) for 16 h at 37 °C. Gels were stained with 0.1% Coomassie Brilliant Blue (Sigma) for 1 h and de-stained with methanol: acetic acid: water (3:1:6, v/v/v) (Merck).

2.10. Detection of mycoviral infection

2.10.1. Cultivation of isolates and nucleic acid extraction for double-stranded RNA detection

Each fungal suspension (10^6 conidia mL^{-1}) was incubated in an Erlenmeyer flask containing 100 mL of Cove liquid medium (0.6% $NaNO_3$, 1% glucose, 0.2% peptone, 0.05% yeast extract and 0.15% casein). Cultures were incubated in an orbital shaker operating at 150 rpm at 28 °C for 72 h. After this period, the mycelium was collected by filtration using sterile Whatman No. 1 filter paper and a vacuum pump. The extraction of total nucleic acid was performed according to the method describe by Zhang et al. (1996).

2.10.2. Enzymatic digestion of total nucleic acid

Single strand RNA was digested using 0.5 μL of S1 nuclease (100 U/μL) (Sigma Chem. Co., St Louis, MO, USA), 0.5 μL of total nucleic acid (∼200 ng), 2 μL of S1 nuclease enzyme buffer (10×) (2.8 M NaCl/300 nM $C_2H_3NaO_2$ pH 4.5/10 mM $ZnSO_4$) and 12.5 μL of DEPC treated Milli-Q water (Sigma). Reactions were incubated at 37 °C for 1 h then plunged in a cold (ice) treatment to stop the reaction (Arruda, 2005). Both single- and double-stranded RNA was digested using 5 μL of total nucleic acid (∼200 ng), 0.2 μL of RNAse A (200 μg/mL) (Invitrogen) and 4.8 μL of DEPC-treated Milli-Q water. Reactions were incubated at 37 °C for 30 min then plunged in a cold (ice) treatment to stop the reaction. The products of these enzymatic treatments were visualized on 1.2% agarose gels stained with 0.5 μg of ethidium bromide; the "1 kb Plus DNA Ladder" (Invitrogen) molecular weight size marker was used. The *M. anisopliae* isolate CG 31 (Cenargen, DF-Brazil) was used as a positive control according to the method of Arruda (2005).

2.11. Statistical analysis

Parametric data (EMW, EPI, NI and enzyme assays) were analyzed by analysis of variance (ANOVA) and the Tukey test. Non-parametric data (larval hatch percent) were assessed by Kruskal–Wallis test and Student–Newman–Keuls test. A significance level of 5% was used (Sampaio, 2002).

3. Results

Fungal suspensions from all isolates used in the experiments described above achieved approximately100% conidial germination after 24 h of incubation in PDA.

Most of the biological parameters of the engorged tick females were altered by all five fungal isolates. The CG 148 and CG 629 isolates reduced the EMW by 58%, while the CG 112 and CG 347 isolates reduced the EMW by 28%. However, the EMW of females treated with the isolate CG 32 was not different ($P > 0.05$) from the EMW of untreated females or females exposed to CG 112 or CG 347 (Table 1).

Table 1
Mean values[a] ± standard deviation of egg mass weight (E.M.W.), larval hatch percent (L.H.P.), egg production index (E.P.I.), nutritional index (N.I.), and control percent (C.P.) of *Rhipicephalus microplus* females treated with fungal aqueous suspension (10^6 conidia mL^{-1}) of five *Metarhizium anisopliae* s.l. isolates. Experiments were conduced at 27 °C and ≥80% RH.

Treatments	E.M.W. (mg)	L.H.P. (%)	E.P.I. (%)	N.I. (%)	C.P. (%)
Control	134.9 ± 0.01^{a}	98.6 ± 0.51^{a}	58.1 ± 2.39^{a}	72.3 ± 5.99^{a}	–
CG 112	93.9 ± 0.02^{b}	91.0 ± 5.67^{b}	43.4 ± 13.46^{b}	52.2 ± 12.8^{b}	30.9
CG 347	105.9 ± 0.02^{b}	83.0 ± 11.10^{b}	45.6 ± 12.18^{ab}	53.4 ± 13.95^{b}	31.5
CG 32	109.0 ± 0.02^{ab}	67.5 ± 2.11^{c}	48.8 ± 11.27^{ab}	57.4 ± 11.82^{b}	43.4
CG 148	62.8 ± 0.02^{c}	83.8 ± 12.59^{bc}	26.15 ± 9.19^{c}	31.1 ± 9.50^{c}	61.5
CG 629	56.1 ± 0.02^{c}	75.0 ± 24.60^{bc}	25.7 ± 8.72^{c}	30.3 ± 9.24^{c}	66.4

[a] Mean values followed by the same letter in the same column do not differ statistically ($P > 0.05$).

The LHP of all isolates was lower in the treated ticks than in the control ticks; the CG 32 isolate reduced this parameter the most (by approximately 32%) (Table 1).

The egg production index is a parameter that represents how much of a female's weight was converted to eggs and compares the initial female weight to the total egg mass weight; the isolates CG 148 and CG 629 reduced this parameter by 53%. The other isolates also reduced this biological parameter but did so to much lower extents (Table 1).

Similar results were obtained when the nutritional index was evaluated. This parameter compares the female's total egg mass weight with the amount of blood ingested by this female. The isolates CG 148 and CG 629 reduced this parameter by approximately 59%. The isolates CG 32, CG 112, and CG 347 also significantly ($P < 0.05$) reduced the nutritional index. Accordingly, because all these parameters are related to the reproductive ability of tick females, the isolates CG 148 and CG 629 achieved the best tick control, resulting in levels of control of 61.5% and 66.9%, respectively. Isolate CG 32 had an intermediate virulence, yielding a tick control of 43.4%; CG 112 and CG 347, which were both considered less virulent, achieved 30% tick control (Table 1).

In the lipolytic assay, the highest levels of enzymatic activity after 24 h of growth were found in the CG 629, CG 32 and CG 148 isolates. After 48 h of growth, the isolate CG 629 had the highest level of lipolytic activity, followed by CG 148 and CG 347. After 72 h, the highest levels of lipolytic activities were found in the CG 629, CG 148, and CG 347 isolates. No discernible patterns in lipolytic activity were observed when the enzymatic activity of each isolate was compared to the time of fungal time cultivation; accordingly, significantly increased enzymatic activity with increasing time of cultivation was observed only for isolate CG 347. Overall, the most virulent isolates against *R. microplus* already displayed high levels of enzymatic activity in the first 24 h of cultivation; in contrast, the less virulent isolate (CG 112) had low levels of enzymatic activity at all times (Table 2).

Protease activity was detected in all five isolates after 24 h of growth; however, the highest level of protease activity was detected in the CG 32 isolate. After 48 h and 72 h of cultivation, the highest levels of lipolytic activity were detected in the CG 148 isolate, followed by the CG 629 and CG 32 isolates. This enzyme assay revealed that there was a progressive increase in protease activity with increasing time of cultivation; in particular, between 24 h and 48 h, a significant difference in protease activity was observed in all isolates (Table 3).

Table 2
Lipase activity (*U*) (μMol/mL/min) of different *Metarhizium anisopliae* s.l. fungal isolates cultivated in liquid medium containing 1% tick cuticle and 1% cholesteryl stearate.[a]

Time of cultivation (h)			
Isolates	24	48	72
CG 112	0.12 ± 0.004^{aA}	0.12 ± 0.002^{aA}	0.12 ± 0.003^{aA}
CG 347	0.13 ± 0.002^{aA}	0.27 ± 0.003^{bB}	0.49 ± 0.008^{bC}
CG 32	0.27 ± 0.011^{bA}	0.10 ± 0.003^{aB}	0.12 ± 0.003^{aB}
CG 148	0.26 ± 0.020^{bA}	0.32 ± 0.004^{bA}	0.33 ± 0.003^{bA}
CG 629	0.39 ± 0.002^{bA}	0.42 ± 0.008^{cA}	0.25 ± 0.008^{bB}

[a] Lipase activity (*U*) followed by the same lower case letter in the same column and the same upper case in the same row do not differ statistically ($P \geq 0.05$).

To evaluate the diversity of proteases synthesized by the different *M. anisopliae* s.l. isolates, *in* gel zymography was performed. According to the molecular weight size markers, the highest protease activities occurred in proteins with sizes of approximately 30 kDa. These enzymes were observed in samples of all isolates that were tested in the present study (Fig. 1). Nevertheless, isolates CG 148 and CG 629 digested the substrate more intensely, thereby displaying higher enzymatic activity; similar results were found in the analysis that used azocasein as a substrate.

The chitinolytic assay showed that after 24 h, the CG148 and CG 629 isolates had significantly higher levels of enzymatic activity than the other isolates. After 48 h and 72 h of cultivation, the CG 629 isolate had the highest level of enzymatic activity, followed by CG 148. The CG 32 isolate displayed intermediate chitinase activity and the CG 112

Table 3
Protease activity (*U*) of different *Metarhizium anisopliae* s.l. fungal isolates cultivated in liquid medium containing 1% tick cuticle and 1% cholesteryl stearate.[a]

Time of cultivation (h)			
Isolates	24	48	72
CG 112	0.20 ± 0.006^{aA}	17.60 ± 0.030^{aB}	19.55 ± 0.029^{aB}
CG 347	0.47 ± 0.002^{aA}	4.75 ± 0.007^{bB}	5.28 ± 0.064^{bB}
CG 32	4.32 ± 0.025^{bA}	27.05 ± 0.134^{acB}	29.08 ± 0.082^{cB}
CG 148	0.57 ± 0.015^{aA}	33.47 ± 0.079^{cB}	36.68 ± 0.055^{cB}
CG 629	0.14 ± 0.001^{aA}	23.56 ± 0.119^{acB}	35.44 ± 0.006^{cC}

[a] Protease activity (*U*) followed by the same lower case letter in the same column and the same upper case in the same row do not differ statistically ($P \geq 0.05$).

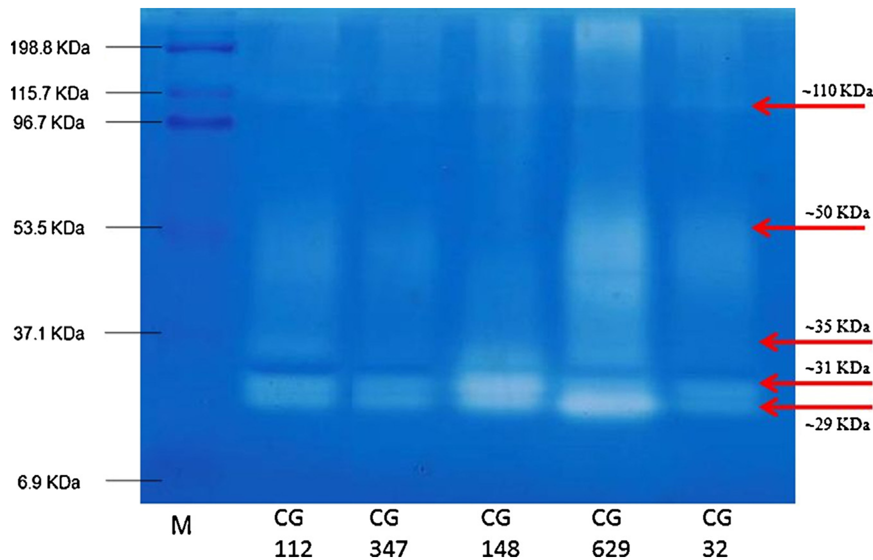

Fig. 1. 12% polyacrylamide *in* gel zymography containing gelatin as substrate. M: molecular weight size markers; fungal isolates CG 112, CG 347, CG 148, CG 629, and CG 32 were cultivated for 72 h.

Table 4
Chitinase activity (*U*) (μMol/mL/min) of different *Metarhizium anisopliae* s.l. fungal isolates cultivated in liquid medium containing 1% tick cuticle and 1% cholesteryl stearate.[a]

	Time of cultivation (h)		
Isolates	24	48	72
CG 112	1.04 ± 0.004^{aA}	1.39 ± 0.002^{aA}	1.77 ± 0.003^{aA}
CG 347	2.83 ± 0.007^{aA}	5.14 ± 0.001^{bB}	7.84 ± 0.002^{bC}
CG 32	5.01 ± 0.002^{bA}	7.56 ± 0.001^{bB}	12.82 ± 0.002^{cC}
CG 148	10.40 ± 0.002^{cA}	12.52 ± 0.001^{cA}	19.47 ± 0.009^{dB}
CG 629	11.88 ± 0.002^{cA}	24.97 ± 0.008^{dB}	27.34 ± 0.003^{eB}

[a] Chitinase activity (*U*) followed by the same lower case letter in the same column and the same upper case in the same row do not differ statistically ($P \geq 0.05$).

and CG 347 isolates displayed the lowest levels of chitinase activity (Table 4).

All isolates were assessed for double-stranded RNA viral infections. Total nucleic acid levels were analyzed by electrophoresis on 1.2% agarose gel; molecules that could migrate faster than chromosomal DNA were observed. dsRNA ranging in size from 1650 bp to 4000 bp was detected. Only the CG 347 isolate was not infected with a dsRNA virus (Fig. 2).

4. Discussion

In this bioassay, engorged *R. microplus* females were infected with five isolates of *M. anisopliae* s.l. and experienced significant alterations in their reproductive parameters. Females that were not treated laid their eggs normally, and all their eggs were determined to be viable under laboratory conditions (27 °C > 80% RH) (Glória et al., 1993). The *in vitro* pathogenicity of *M. anisopliae* s.l. in ticks has been previously reported (Bittencourt et al., 1992; Perinotto et al., 2012); negative effects of this fungus on reproductive parameters of *R. microplus* were observed, resulting in a reduction in the total egg mass weight and in larval hatching. This decreased reproductive capacity and the reduction in larval hatching promote tick control, as both reduce the number of larvae infesting the field.

The current study reported differences in tick control achieved by the different *M. anisopliae* s.l. isolates. Variations in fungal virulence in ticks for the same fungal species have been reported (Quinelato et al., 2012; Leemon and Jonsson, 2008; Ángel-Sahagún et al., 2010; Fernandes et al., 2011). Previous studies have attributed this variation to several factors, including the geographical origin of the isolate, the substrate from which the fungus was isolated (Vicentini et al., 2001), genetic variability (Fernandes et al., 2011), passages in artificial medium (Fargues and Robert, 1983), host susceptibility (Perinotto et al., 2012; Fernandes et al., 2011), differences in the secretion of proteases, lipases and chitinases (Mustafa and Kaur, 2009) and infection by mycoviruses (Frazzon et al., 2000). Accordingly, tests to select more virulent isolates become increasingly necessary, as the choice of fungal strain directly impacts the control of the target pest.

In general, the *M. anisopliae* s.l. isolates tested in the present study that achieved the best control of *R. microplus* ticks also had the highest levels of hydrolytic activity of enzymes directly involved in the fungal infection process. In a similar study using *Beauveria bassiana* isolates, Pelizza et al. (2012) reported that fungal isolates that displayed the highest levels of chitinase activity were more pathogenic to *Tropidacris collaris* (Orthoptera: Romaleidae); these authors recommended using chitinase activity assays to select the *B. bassiana* isolates that were most virulent in grasshoppers. Measurements of enzymatic activities in *M. anisopliae* s.l. have been extensively studied to explain host-pathogen interactions and the role of each enzyme group in the fungal infection process (Silva et al., 2005; Boldo et al., 2009; Beys da Silva et al., 2010a,b; Santi et al., 2010a,b). Enzymatic assays conducted in the present study

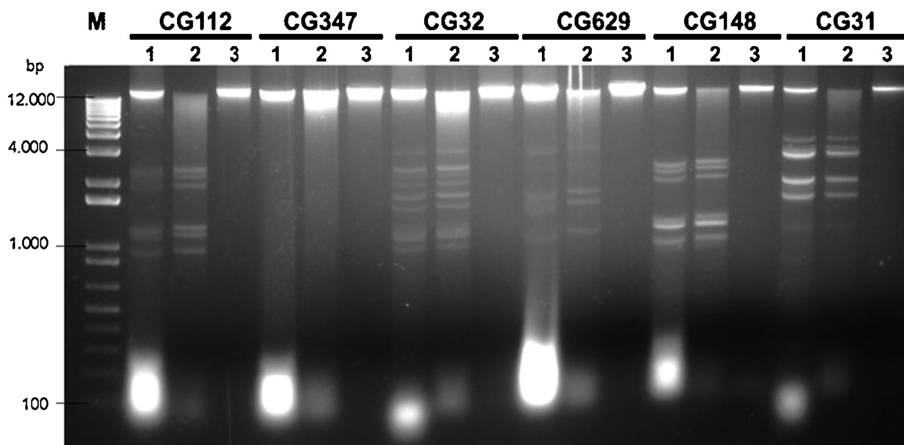

Fig. 2. 1.2% agarose gel electrophoresis of total nucleic acid from different *Metarhizium anisopliae* s.l. isolates. M: molecular weight size markers (1 kb Plus DNA Ladder – Promega); 1: total nucleic acid with no treatment; 2: endonuclease S1-treated nucleic acid; 3: RNAse A-treated nucleic acid; Isolate 31 was used as positive control.

showed that the lipolytic, proteolytic and chitinolytic activities varied among the tested fungal isolates. Similar results were reported by St Leger et al. (1986) who analyzed the activity of these enzymes produced by *M. anisopliae* isolates grown in liquid cultures containing comminuted locust cuticle as a carbon source. According to these authors, the variation in enzymatic activity is thought to be due to differences in the rate of growth of fungal isolates and the consequent lower or higher amounts of enzyme released to hydrolyze substrates and obtain nutrients for fungal development.

According to Beys da Silva (2009), the hydrophobic character of lipases and fatty acids released during lipid hydrolysis could facilitate the adhesion process of the fungi on arthropods. Furthermore, lipids present in the epicuticle are hydrolyzed to provide nutrients for conidia germination and to enable the recognition of the host. In the present study, the isolates that achieved the best tick control also had the highest levels of lipase activity after only 24 h of growth. This result is in accordance with findings of Beys da Silva et al. (2010a) who demonstrated that lipases secreted by *M. anisopliae* s.l. were involved in the early stages of penetration; also, the outer face of the arthropod cuticle (the epicuticle) consists primarily of lipids (Santi et al., 2010b).

The proteolytic assay showed that the isolates that were more virulent to *R. microplus* also exhibited the highest levels of proteolytic activity. A study conducted by Gupta et al. (1994) aimed to determine the association between *Galleria mellonella* larvae mortality caused by *B. bassiana* isolates and the activities of these fungal proteases; these authors reported that the isolates with the highest levels of protease activity were more virulent to *G. mellonella*. The association between protease activity and time of fungal growth reported in the present study is consistent with findings of Arruda et al. (2005) who demonstrated that *M. anisopliae* s.l. invasion in ticks occurs to a greater extent 72 h after contact.

The enzymatic activity of the fungal proteolytic complex is closely related to the hydrolysis of the procuticle proteins of the host (St Leger et al., 1986). Previous studies investigated the presence of extracellular enzymes on the surface of fungal conidia and reported that there are multiple isoforms of proteases; one of these is an approximately 30-kDa-sized subtilisin-like protease referred to as Pr1 (Santi et al., 2010a). This protease is known to be involved in the penetration of host arthropods. This enzyme hydrolyzes approximately 30% of all proteins in the procuticle and is therefore considered to be important for *M. anisopliae* s.l. virulence (St Leger et al., 1996). In the present study, results from gel zymography showed bands with molecular masses corresponding to approximately 30 kDa (which is similar to the molecular weight of Pr1); the highest levels of activity were found in the most virulent isolates (CG 148 and CG 629). Accordingly, it is thought that the activity of these serine proteases directly impact the virulence of the isolates in ticks.

In *M. anisopliae* s.l. isolates, a direct association between higher chitinase activity and higher virulence in female ticks was observed. Furthermore, a progressive increase in chitinase activity with increasing time of growth was observed. According to St Leger et al. (1996), *M. anisopliae* secretes several chitinases that are involved in both fungal growth and the degradation of the arthropod cuticle; this explains why significantly increased levels of chitinase activity were associated with longer fungal cultivation times. Chitinases are enzymes involved in the hydrolysis of chitin present in the procuticle of arthropods; because proteins and chitin are present in the same portion in the procuticle, it is thought that the action of chitinases and proteases occurs concurrently. Synchronicity in the action of these enzymes is supported in the current study by the fact that the highest levels of chitinolytic and proteolytic activity occurred after 48 h and 72 h of growth.

In addition to investigating fungal enzymatic activities, we sought to determine the association between mycoviral infection and the virulence of AP fungi. The results of the current study suggest that the infection of *M. anisopliae* s.l. fungi with dsRNA viral particles is not associated with the virulence of these fungi in *R. microplus*; indeed, both the most virulent isolates and the least virulent isolates were infected with dsRNA viral particles. Similar results were found by Martins et al. (1999) and Arruda

(2005) who found no association between the presence of viral dsRNA particles and *Metarhizium* spp. virulence. According to Day et al. (1977) mycoviral infection in phytopathogenic fungi is closely related to fungal virulence, colony morphology, colony color and the size of conidia. However, Melzer and Bidochka (1998) concluded that the mycoviral infection might be latent in some fungal species and therefore does not result in any difference in fungal morphology. The isolates used in this study had been morphologically analyzed by Quinelato et al. (2012); these authors reported no difference in the size of conidia. Mycoviruses may interfere with the secretion of hydrolytic enzymes and therefore may affect the virulence of AP fungi. According to Giménez-Pecci et al. (2002), chitinase activity was lower in all *M. anisopliae* s.l. isolates that were infected with mycoviruses than in dsRNA-free isolates. In the present study, no association between mycoviral infection and the activity of hydrolytic enzymes was observed, as the isolates with the highest enzymatic activities were positive for viral particles. However, more studies using a greater number of isolates are required to verify the effect of viral particles on the behavior of AP fungi.

The results observed in this work reinforce the hypothesis that enzymes produced by *M. anisopliae* s.l. are important for the fungal infection process in cattle ticks; this study also described the temporal sequence in which these hydrolases are secreted. Accordingly, the quantification of enzymatic activities is recommended as a useful tool for identifying biochemical markers of *M. anisopliae* s.l. virulence in *R. microplus*, especially because these analyses are quick, easily reproducible and inexpensive. Finally, the GC 148 and GC 629 isolates should be used in future tests aiming to control *R. microplus* ticks.

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