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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.

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 $25 \pm 1\,^{\circ}C$ and $\geq$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 ($\sim$50 μL) of conidial suspensions on PDA and incubating the sample at $25 \pm 1\,^{\circ}C$ and $\geq$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 $27\,^{\circ}C$ and $\geq$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 $27 \pm 1\,^{\circ}C$ and $\geq$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 $25\,^{\circ}C$. 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 $-80\,^{\circ}C$.