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# Effect of heat stress and oil formulation on conidial germination of *Metarhizium anisopliae* s.s. on tick cuticle and artificial medium

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ABSTRACT

The effect of heat stress (45 °C) versus non-heat stress (27 °C) on germination of *Metarhizium anisopliae* sensu stricto (s.s.) isolate IP 119 was examined with conidia formulated (suspended) in pure mineral oil or in water (Tween 80, 0.01%), and then applied onto the cuticle of *Rhipicephalus sanguineus* sensu lato (s.l.) engorged females or onto culture medium (PDAY). In addition, bioassays were performed to investigate the effect of conidia heated while formulated in oil, then applied to blood-engorged adult *R. sanguineus* females. Conidia suspended in water then exposed to 45 °C, in comparison to conidia formulated in mineral oil and exposed to the same temperature, germinated less and more slowly when incubated on either PDAY medium or tick cuticle. Also, conidial germination on tick cuticle was delayed in comparison to germination on artificial culture medium; for example, germination was 13% on tick cuticle 72 h after inoculation, in contrast to 61.5% on PDAY medium. Unheated (27 °C) conidia suspended in either water or oil and applied to tick cuticle developed appressoria 36 h after treatment; whereas only heat-stressed conidia formulated in oil developed appressoria on tick cuticle. In comparison to conidia heated in mineral oil, there was a strong negative effect of heat on germination of conidia heated in water before being applied to arthropod cuticle. Nevertheless, bioassays [based primarily on egg production (quantity) and egg hatchability] exhibited high percentages of tick control regardless of the type of conidial suspension; i.e., water- or oil-formulated conidia, and whether or not conidia were previously exposed to heat. In comparison to aqueous conidial preparations, however, conidia formulated in oil reduced egg hatchability irrespective of heat or no-heat exposure. In conclusion, mineral-oil formulation protected conidia against heat-induced delay of both germination and appressorium production when applied to the cuticle of *R. sanguineus*.



## 1. Introduction

The brown dog tick, *Rhipicephalus sanguineus* Latreille, 1806 (Acari, Ixodidae), is widely distributed throughout the world; and it is considered one of the main ectoparasites of domestic dogs. Besides its preference for dogs, *R. sanguineus* occasionally parasitizes other domestic and wild animals, including humans (Estrada-Peña and Jongejan, 1999; Dantas-Torres et al., 2006; Dantas-Torres, 2008, 2010; Louly et al., 2006).

*R. sanguineus* vectors many dog pathogens such as *Babesia canis* and *Ehrlichia canis* (Dantas-Torres, 2008), and high infestations, even without vectoring pathogens, may cause skin irritation, anemia and lethargy. In spite of its low anthropophily (Palmas et al., 2001), it is known to trigger inflammatory or allergic reactions to the bites of larvae and nymphs. Transmission of pathogens to humans by *R. sanguineus* is well documented; for example: it is an important vector of the bacterium *Rickettsia conorii*, the etiologic agent of Mediterranean spotted fever or Boutonneuse fever in Europe (Merle et al., 1998; Matsumoto et al., 2005); and *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in the United States (Demma et al., 2005) and Mexico (Eremeeva et al., 2011). In Brazil, *R. sanguineus* is considered a potential vector to humans of *R. rickettsii* (Rozental et al., 2002),

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agent of the Brazilian spotted fever (Rocky Mountain spotted fever in North America), and *Borrelia* sp., agent of Lyme-like disease (Yoshinari et al., 1997). Laboratory tests confirmed that *R. sanguineus* is a competent vector of *R. rickettsii* (Piranda et al., 2011), and molecular studies and isolation in cell culture demonstrated natural infection of this tick with *R. rickettsii* in endemic areas of Brazilian spotted fever in Brazil (Cunha et al., 2009; Ogrzewalska et al., 2012).

The control of *R. sanguineus* is mainly based on the use of chemical acaricides applied directly on infested dogs and/or in the dogs' environments (Labruna and Pereira, 2001; Dantas-Torres and Figueredo, 2006). However, over the years, many tick populations developed tolerance to most chemicals used. Misuse, e.g., mistakes during preparation of the product, inappropriately low application rates, and overly repeated applications of chemical acaricides, are situations that contributed to the selection of chemical-acaricide-resistant tick populations (Labarthe, 1994). Resistance of *R. sanguineus* against chemical products has been demonstrated worldwide (Burridge et al., 2004; Miller et al., 2001; Estrada-Penã, 2005), as well as in Brazil (Borges et al., 2007).

Tick pathogens, in particular entomopathogenic fungi, have been investigated as promising biocontrol alternatives to the current widespread excessive and almost exclusive use of chemicals for tick control. The objectives of fungus studies are to reduce both acaricides resistance and environmental contamination. Fungi have been recognized repeatedly as the most promising microorganisms for microbial control of ticks, e.g., *Rhipicephalus microplus* (Bittencourt et al., 1999ab; Arruda et al., 2005; Fernandes and Bittencourt, 2008; Leemon and Jonsson, 2008); *R. sanguineus* (Samish et al., 2001; Prette et al., 2005; Garcia et al., 2004; Garcia et al., 2005); *Amblyomma cajennense* s.l. (Souza et al., 1999; Reis et al., 2001; D'Alessandro et al., 2012); *Amblyomma cooperi* (Reis et al., 2003); and *Amblyomma variegatum* (Kaaya et al., 1996; Maranga et al., 2005).

Abiotic factors, especially high temperatures, may be important obstacles to the effectiveness of entomopathogenic fungi applied for arthropod-pest control. High temperatures may reduce the virulence and/or the survival of these microorganisms; and, consequently, limit their effectiveness (Fernandes et al., 2010; Rangel et al., 2010; Ment et al., 2011). The optimum temperature range for *Metarhizium anisopliae* s.l. is between 24 and 30 °C (Walstad et al., 1970; Dimbi et al., 2004). Rangel et al. (2010) reported that several *Metarhizium* isolates germinated well at constant 35 °C, but mycelial growth was very slow at 36 °C in comparison to growth at 28 °C; also, the exposure of different isolates to 37 °C for 96 h (Santos, 1978) or 45 °C for 8 h (Fernandes et al., 2010) totally inhibited conidial germination. Nevertheless, *Metarhizium* species and isolates may vary greatly in thermotolerance. For example, heat-treated conidia may undergo significantly delayed germination, depending mostly on the fungal species, isolate and the heat-exposure time (Rangel et al., 2005; Fernandes et al., 2010; Keyser et al., 2014). Of particular interest in this regard is the markedly elevated thermotolerance of all *M. acridum* isolates tested to date (Fernandes et al., 2010).

The temperature, as expected, may vary considerably from one locality to another; e.g., in dog kennels and enclosures, high temperatures may be reached at least for a few hours a day due to direct or indirect sunlight. High temperatures may reduce the viability of conidia (bioproduct) applied locally for tick control, and also may seriously influence the biology of ticks (Bellato and Daemon, 1997a,b), e.g., reducing the molting periods of larvae and nymphs, longevity of nymphs and adults, period of preoviposition and oviposition of engorged females, and length of egg incubation periods, i.e., eggs incubated at high temperature (32 °C) hatched quicker than those held at low (18 °C) or optimum (27 °C) temperature (Bellato and Daemon, 1997a).

Formulation is a much studied approach to protecting fungal propagules from environmental adversities and, thereby, increase their efficiency as biocontrol agents of arthropods. The combination of oil and conidia, for example, assists in protecting the fungus against rapid dehydration in low-humidity environments (Bateman et al., 1993; Bateman, 1994); high temperatures (McClatchie et al., 1994); and ultraviolet radiation (Moore et al., 1993; Alves et al., 1998; Bateman and Alves, 2000). In comparison with conidia conventionally prepared in water, oil-based formulations favor the spreading and adhesion of conidia on the hydrophobic cuticle of host arthropods (David-Henriet et al., 1998). In addition, oil extends the viability of conidia held at ambient conditions or under refrigeration (Prior et al., 1988). The positive effects of formulating entomopathogenic fungi in oil have been reported as, (a) increased fungus-induced mortality against cocoa weevil (Prior et al., 1988), triatomine bugs (Luz and Batagin, 2005), grasshoppers (Lomer et al., 2001) and whiteflies (Malsam et al., 2002); (b) increased ovicidal activity against *Aedes aegypti* mosquito (Albernaz et al., 2009) and *Rhipicephalus annulatus* tick (Samish et al., 2014), and (c) increased virulence against different life stages of the tick *R. microplus* (Angelo et al., 2010; Camargo et al., 2012).

Unfortunately, the information currently available on conidial thermotolerance was generated using *in vitro* studies, with viability being assessed by germination/cultivation of heat-treated conidia on artificial culture media, rather than observing them on the cuticle of ticks or other arthropods. The research reported here, therefore, assessed the effect of heat stress on germination (a) when conidia of *M. anisopliae* sensu stricto IP 119 were suspended in pure mineral oil or water (Tween 80, 0.01%), then heated or not heated, and finally applied onto the cuticle of *R. sanguineus*. In addition, as a comparison with previous heat-tolerance studies, (b) we investigated the performance of conidia heat-treated in water and then incubated on artificial medium (PDAY). We also assessed (c) the effect of heat treatment of *M. anisopliae* conidia on their appressorium formation on *R. sanguineus* engorged female cuticle; and we (d) compared responses (primarily egg production and egg hatchability) of ticks following exposure to heat-treated (45 °C) and non heat-treated (27 °C) conidia.

## 2. Material and methods

### 2.1. Fungal isolate, cultivation, preparation of conidial suspensions, and assessment of conidial viability

The fungal isolate used in this study was *M. anisopliae* s.s. IP 119, which was obtained from Brazilian Savanna (Cerrado) soil collected from Goiás State, Center-West Brazil and identified by Rocha et al. (2012). IP 119 has been deposited in the Fungal Research Collection of the Laboratory of Invertebrate Pathology, at the Institute of Tropical Pathology and Public Health of the Federal University of Goiás; and, at Embrapa Collection of Entomopathogenic Fungi, Brasília, Brazil, under the code name CG 764. This isolate was selected from a group of ten Brazilian *Metarhizium* isolates, due to its exceptional virulence against *R. microplus* engorged females when conidia were formulated in oil, and moderate virulence when suspended in Tween 80® 0.01% (Muniz and Fernandes, unpublished data).

IP 119 was cultured on 23 mL potato dextrose agar medium (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g $L^{-1}$ yeast extract (Technical, Difco) (PDAY) in glass Petri plates (90 × 15 mm) in the dark at 27 ± 1 °C for 15 days. Conidia were dislodged with a microbiological loop, harvested, weighed, and suspended in Tween 80® (Labsynth Prod. Lab. Ltda, Diadema, SP, Brazil) solution (0.01% v/v) or pure mineral oil (Impex, Labimpex Ind. e Com. de Prods. p/ Lab. Ltda., Diadema, SP). Aqueous conidial suspensions were quantified by hemocytometer counts, and