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Virulence of Mexican isolates of entomopathogenic fungi (Hypocreales: Clavicipitaceae) upon *Rhipicephalus* = *Boophilus microplus* (Acari: Ixodidae) larvae and the efficacy of conidia formulations to reduce larval tick density under field conditions

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Ángel-Sahagún, César Andrés; Lezama-Gutiérrez, Roberto; Molina-Ochoa, Jaime; Pescador-Rubio, A.; Skoda, S. R.; Cruz-Vázquez, C.; Lorenzoni, A. G.; Galindo-Velasco, E.; Fragoso-Sánchez, H.; and Foster, John E., "Virulence of Mexican isolates of entomopathogenic fungi (Hypocreales: Clavicipitaceae) upon *Rhipicephalus* = *Boophilus microplus* (Acari: Ixodidae) larvae and the efficacy of conidia formulations to reduce larval tick density under field conditions" (2010). *Faculty Publications: Department of Entomology*. 625.
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Veterinary Parasitology 170 (2010) 278–286

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Virulence of Mexican isolates of entomopathogenic fungi (Hypocreales: Clavicipitaceae) upon *Rhipicephalus* = *Boophilus microplus* (Acari: Ixodidae) larvae and the efficacy of conidia formulations to reduce larval tick density under field conditions

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ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form 17 February 2010
Accepted 24 February 2010

Keywords:
Biological control
Wheat bran
Fungal formulations
Ticks

ABSTRACT

The first objective was laboratory evaluation of the virulence of 53 Mexican isolates of fungi against larvae of *Rhipicephalus* (*Boophilus*) *microplus*. Thirty-three isolates of *Metarhizium anisopliae* var. *anisopliae* (Metschnickoff) Sorokin (Hypocreales: Clavicipitaceae) and 20 isolates of *Isaria* (*Paecilomyces*) *fumosorosea* (*fumosoroseus*) (Wize) (Eurotiales: Trichomaceae) were tested on 7-day-old larvae under laboratory conditions. Larvae were immersed in a suspension containing 10^8 conidia/mL and the CL_{50} values were estimated. Then, field tests were conducted to determine the efficacy of formulations of the isolate with the highest virulence. *M. anisopliae* (Ma 14 isolate) was formulated with four carriers: Tween, Celite, wheat bran, and Citroline (mineral oil) and applied on pasture beds of *Cynodon plectostachyus* (L.), at a dose of 2×10^9 CFU/m^2. In the first trial, *M. anisopliae* was applied on plots naturally infested with larvae; in the second trial, tick populations in the experimental plots were eliminated and then re-infested with 20,000

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0304-4017/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2010.02.037

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7-day-old larvae. In the laboratory, all *M. anisopliae* isolates infected larvae with a mortality range between 2 and 100%; also, 13 of 20 *I. fumosorosea* isolates caused mortality rates between 7 and 94%. In the first field trial, 14 days post-application, conidial formulations in Celite and wheat bran caused 67.8 and 94.2% population reduction, respectively. In the second trial, the Tween formulation caused the highest larval reduction, reaching up to 61% (28 days post-application). Wheat bran formulation caused 58.3% larval reduction (21 days post-application) and was one of the most effective. The carriers and emulsifiers have a large impact on the effectiveness of conidial formulations.

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1. Introduction

Ticks are blood-sucking organisms that cause great damage to livestock through skin damage that reduces the value of hides for leather, blood loss, behavioural changes leading to decreased weight gains, reduced milk production, and disease transmission (Jonsson et al., 1998; Solorio et al., 1999). Worldwide it has been estimated that ticks and tick-borne diseases cause annual losses between $13.9 and $18.7 billion dollars (De Castro, 1997).

Rhipicephalus (*Boophilus*) *microplus* (Canestrini) (Acari: Ixodidae) is the most important ectoparasite in tropical and subtropical countries of Latin America (Muro et al., 2003; Estrada-Peña et al., 2006a,b) including Mexico (Alonso-Díaz et al., 2007). Acaricides, long the main control source of tick populations in most countries around the world (Polar et al., 2005a,b), have disadvantages including high costs, development of heritable resistance to chemical control (Kunz and Kemp, 1994), and contamination of the environment and feed with residues (Norval et al., 1992). In Mexico, *R. microplus* developed resistance to organophosphates in the 80s, to pyrethroids in the 90s (Santamaría et al., 1999; Miller et al., 1999), and most recently to Amitraz (Soberanes et al., 2002). Due to the resistance of *R. microplus* to several pesticides, biological control seems an appropriate alternative that may reduce the frequency of chemical acaricide use and the need for treatment for tick-borne diseases (Bittencourt et al., 1997a,b; Kaaya and Hassan, 2000; Jonsson et al., 2000; Samish et al., 2001).

Fungi such as *Metarhizium anisopliae* (Metschnickoff) Sorokin, *Cordyceps* (*Beauveria*) *bassiana* (Balsamo) Vuillemin, *Lecanicillium lecanii* (=*Verticillium lecanii*) (Zimmermann) Zare and W. Gams, and *Paecilomyces* = *Isaria fumosorosea* (Wize) Brown and Smith have been studied as control agents of ticks (Bittencourt et al., 1992; Souza et al., 1999; Kaaya and Hassan, 2000; Bittencourt, 2000; Gindin et al., 2001; Samish et al., 2001, 2004a,b; Fernandes et al., 2003, 2006a,b; Fernández-Ruvalcaba et al., 2005; Polar et al., 2005a,b; Beltrán et al., 2008). Studies on the potential of entomopathogenic fungi as tick control agents have been conducted mainly as laboratory assays (Polar et al., 2005c; Leemon and Jonsson, 2008). *M. anisopliae* and *B. bassiana* are known to cause high mortality in various life stages of *R. microplus* (Bittencourt et al., 1992, 1994a,b, 1996, 1997a,b; Samish and Rehacek, 1999). However, studies of fungal effects on ticks under field conditions are scarce (Benjamin et al., 2002; Bittencourt et al., 2003; Basso et al., 2005; Alonso-Díaz et al., 2007). In Latin America field tests have been reported showing promising results; in Brazil, Bittencourt et al. (2003) found 53.78% reduction of *R. microplus* larvae after the application of *M. anisopliae* on pasture beds of *Brachiaria decumbens* Stapf using a concentration of 10^9 conidia/mL in water suspension. More recently, Basso et al. (2005) evaluated the effect of *M. anisopliae* applied in a water suspension against *R. microplus* larvae and reported reduction of larvae ranging between 88 and 94.08%.

Conidial formulations tested so far are water based containing a small amount of dispersing agent (Samish et al., 2004a,b) but there is evidence that oil-based formulations have been successful against several insect pests (Prior et al., 1988; Lomer et al., 1992; Bateman et al., 1993). Consequently, it is necessary to evaluate the effects of a variety of fungal formulations on tick mortality. This manuscript summarizes several studies to determine the virulence of 33 Mexican isolates of *M. anisopliae* and 20 isolates of *I. fumosorosea* upon *R. microplus* larvae under laboratory conditions. Also, reported is the efficacy of solid- and liquid-based conidia formulations of the most virulent isolate against *R. microplus* larvae to reduce tick larval density under field conditions.

2. Materials and methods

2.1. Tick colony

Engorged female *R. microplus* ticks were collected from naturally infested cattle at the ranch "La Guadalupana" located in Coquimatlán, Colima, Mexico (19°09′31.2″N; 103°50′12.5″W). The ticks were washed in distilled water, dried, and placed in Petri dishes lined with a layer of filter paper (Whatman No. 1) moistened with sterile distilled water. The plates were incubated deprived of light in an environmental chamber (Forma Scientific, Inc. Mod 3740, OH, USA) at 25 ± 1 °C with a relative humidity ≥ 80%. After oviposition, eggs were transferred into different Petri dishes and incubated until larval emergence. Tick larvae were maintained in the environmental chamber for 7 days before laboratory bioassays (Gindin et al., 2001, 2002).

A second group of *R. microplus* engorged female ticks were treated as described above to obtain additional egg masses. Within 1 week from the oviposition, eggs were collected to form 1-g aliquots (∼20,000 larvae). The egg aliquots were then placed in 2 mL tubes, sealed with hydrophilic cotton and incubated until larval emergence (Bahiense et al., 2006). Seven day-old tick larvae were used in a field experiment described in Section 2.6.

Table 1
Metarhizium anisopliae (Ma) and *I. fumosorosea* (Ifr) isolates used in the present work.

Fungus isolate	Host or substrate	Geographical origin	Date
Ma 2	*Diatraea saccharalis* Fabr. (Lepidoptera: Pyralidae)	Colima, Mexico	1988
Ma 3	*D. saccharalis*	Colima, Mexico	1987
Ma 4	*D. saccharalis*	Colima, Mexico	1987
Ma 5	*Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae)	Colima, Mexico	1988
Ma 6	*S. frugiperda*	Colima, Mexico	1989
Ma 7	*Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae)	Tamaulipas, Mexico	1987
Ma 8	*Geraeus seniles* Gyllenhal (Coleoptera: Curculionidae)	Colima, Mexico	1990
Ma 9	Soil	Colima, Mexico	1993
Ma 10	*Macrodactylus murinus* Bates (Coleoptera: Scarabaeidae)	Colima, Mexico	1993
Ma 12	Soil	Colima, Mexico	1993
Ma 13	Soil	Colima, Mexico	1993
Ma 14	*M. murinus*	Colima, Mexico	1993
Ma 15	*Schistocerca piceifrons* Walter (Orthoptera: Acrididae)	Colima, Mexico	1993
Ma 16	*Conotrachelus perseae* Barber (Coleoptera: Curculionidae)	Michoacan, Mexico	1999
Ma 22	*Aeneolamia postica* (Walker) (Homoptera: Cercopidae)	Colima, Mexico	2003
Ma 23	*A. postica*	Veracruz, Mexico	1994
Ma 26	*S. piceifrons*	Colima, Mexico	1993
Ma 28	*S. piceifrons*	Colima, Mexico	1999
Ma 29	*S. piceifrons*	Colima, Mexico	1993
Ma 30	Soil	Jalisco, Mexico	2000
Ma 34	Soil	Michoacán, Mexico	2000
Ma 35	Soil	Jalisco, Mexico	2000
Ma 37	Soil	Nayarit, Mexico	2000
Ma 40	Soil	Zacatecas, Mexico	2000
Ma 41	Soil	Zacatecas, Mexico	2000
Ma 46	Soil	Colima, Mexico	2000
Ma 47	Soil	Colima, Mexico	2000
Ma 49	Soil	Colima, Mexico	2000
Ma 50	Soil	Colima, Mexico	2000
Ma 51	Soil	Colima, Mexico	2000
Ma 54	Soil	Colima, Mexico	2000
Ma 58	Soil	Colima, Mexico	2000
Ma 59	Soil	Colima, Mexico	2000
Pfr 1	*Bemisia tabaci* (Genn.) (Homoptera: Aleyrodidae)	Colima, Mexico	1991
Pfr 2	*B. tabaci*	Colima, Mexico	1991
Pfr 3	*B. tabaci*	Colima, Mexico	1994
Pfr 4	*B. tabaci*	Colima, Mexico	1994
Pfr 5	*B. tabaci*	Sinaloa, Mexico	1995
Pfr 6	*B. tabaci*	Colima, Mexico	1994
Pfr 7	*B. tabaci*	Colima, México	1994
Pfr 8	*S. piceifrons*	Colima, Mexico	1992
Pfr 9	*S. piceifrons*	Colima, Mexico	1992
Pfr 10	*Toxoptera aurantii* (Boyer de Fonscolombe) (Homoptera: Aphididae)	Colima, Mexico	1998
Pfr 11	*B. tabaci*	Colima, Mexico	1994
Pfr 12	*S. piceifrons*	Colima, Mexico	1992
Pfr 13	*T. aurantii*	Colima, Mexico	2000
Pfr 14	*B. tabaci*	Yucatan, Mexico	1990
Pfr 15	*B. tabaci*	Yucatan, Mexico	1990
Pfr 16	*B. tabaci*	Yucatan, Mexico	1990
Pfr 17	*B. tabaci*	Yucatan, Mexico	1990
Pfr 18	*B. tabaci*	Yucatan, Mexico	1990
Pfr 19	Soil	Colima, Mexico	2004
Pfr 20	Soil	Colima, Mexico	2005

2.2. Entomopathogenic fungi isolates and culture conditions

Fungal isolates used in this study were obtained from the Mycological Collection of the Universidad de Colima, Facultad de Ciencias Biológicas y Agropecuarias, Tecomán Campus, Colima, Mexico. Thirty-eight isolates were collected in the State of Colima, and 15 were obtained from other Mexican states (Table 1). Among these, 33 were identified as *M. anisopliae* and 20 as *I. fumosorosea*. The isolates, obtained from the field over the last 20 years and kept under laboratory conditions, were reproduced in Sabouraud dextrose agar medium enriched with 1% yeast extract (Watson et al., 1995) and containing 500 ppm of chloramphenicol (Sneh, 1991). Isolates were incubated for 21 days (Arthurs and Thomas, 2001) at 25 ± 1 °C (Barson et al., 1994) under a 12:12 h light/darkness. Conidia from each isolate were harvested by scraping and then suspended in sterile distilled water containing 0.1% (v/v) Tween 80 (Samuels et al., 2002). The conidial suspension was poured into a sterile glass tube and vortexed for spore viability determination (Lezama-Gutiérrez et al., 2000).

Table 2
Ingredients of the experimental formulations for the field experiment, "Rancho el Peregrino" 2007, Colima, Mexico.

Treatments	Constituents	Specifications
Control	Water	Tween 80 (0.1%) (v/v)
	Tween	Agricultural surfactant (1%) (Inex-A; Cosmocel, S. A.)[a]
	Agricultural surfactant	
Tween	Conidia	2×10^{13} conidia/ha
	Water	
	Tween	Tween 80 (0.1%) (v/v)
	Agricultural surfactant	Agricultural surfactant (1%) (Inex-A; Cosmocel, S. A.)
Citroline	Conidia	2×10^{13} conidia/ha
	Water	
	Tween	Tween 80 (0.1%) (v/v)
	Agricultural surfactant	Agricultural surfactant (1%) (Inex-A; Cosmocel, S. A.)
	Mineral oil	Citroline (1%) (mineral oil derived from petroleum for agricultural use in Mexico)
Celite	Conidia	2×10^{13} conidia/ha
	Celite	Diatomaceous earth (Celite 209, Celite Corp.) (50 kg/ha).
	Water	(Only in the second trial)
Wheat bran	Conidia	2×10^{13} conidia/ha
	Wheat bran	50 kg/ha

[a] Contents of Inex-A (Cosmocel, S. A., México): polyethylene glycol 5.2%, glycol with ethylene oxide 20.6%, dimethyl polysiloxane 1.85% and the inert ingredients (carrier) 72.35%.

2.3. Spore viability determination

Conidia concentrations were determined by direct count using an improved Neubauer hemocytometer (Reichert Scientific Instruments, Buffalo, New York). One millilitre of each conidial suspension was adjusted to 10^6 conidia/mL by diluting with 0.1% (v/v) Tween 80 (Garcia et al., 2005). Conidia viability was determined by seeding 100 μl of the conidial suspension on Sabouraud dextrose agar and counting colonies 48 h later (Lacey et al., 1994).

2.4. Laboratory bioassays

Pathogenic activity of each isolate was evaluated by adjusting the conidia concentration to 1×10^8 CFU (colony forming units)/mL. Virulence was evaluated by LC_{50} estimation only for the isolates that promoted more than 90% tick mortality. The virulence assays were conducted using seven conidia concentrations: 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 conidia/mL (Arthurs and Thomas, 2001). In all treatments the experimental unit was a Petri dish containing 25 larvae. Experimental ticks were placed on a 5 cm adhesive tape strip and then immersed for 5 s in a specific conidial concentration (Kaaya et al., 1996). Treated ticks were kept in Petri dishes, on a double layer of filter paper (Whatman No. 1) soaked with sterile distilled water and incubated at 25 ± 1 °C under a 12:12 h light/darkness photoperiod. Larval mortality was evaluated every 48 h and fungal infection was confirmed upon fungal sporulation (Smith et al., 2000).

A complete randomized design was used to statistically evaluate pathogenic activity. A total of 53 fungi isolates and a control treatment (four replicates each) were included in the analysis. The mortality rate data were converted using the arcsine transformation before analysis. Data were analyzed using one-way ANOVA (SAS Institute, 1997), and Tukey's test was used to separate treatment means when differences where significant. The virulence data were examined by Probit Analysis to obtain the LC_{50} values (Gindin et al., 2001).

2.5. Conidia mass production for field experiments

The isolate that presented the highest virulence against *R. microplus* in the laboratory bioassays was mass-produced on rice grain. In a previous study, rice was found to be the best media for mass-culturing fungi for field studies (Lezama-Gutiérrez and Munguía, 1990). Polyethylene bags containing 200 g of rice (Miracle rice cultivar) were soaked with distilled water containing 500 ppm of chloramphenicol for 40 min, and autoclaved for 15 min at 121 °C and 15 psi. Each bag was inoculated with 1 mL of a 10^8 conidia/mL suspension with a sterilized syringe and the puncture sealed with sticky tape. Spores were incubated for 3 weeks in the dark at 25 °C (Alonso-Díaz et al., 2007). Finally, conidia were harvested from rice grain using two sieves, Tyler equivalent 40 and 200 meshes (USA. Standard Testing Sieve, A.S.T.M.E-11 Specification, W.S. Tyler, USA) for the first and second passes, respectively. Thereafter, conidia were suspended in distilled water containing 0.1% (v/v) Tween 80, centrifuged at 2200 × g for 15 min (Hanil Science Industrial Co. LTD, Mod. MF10622002) and dried in the dark in a Class II Biosafety Cabinet (Labconco, Missouri, USA) for 8 days at 25 °C. Conidia were kept in the dark at 4 °C until utilized in field trials. Before evaluation under field conditions the final conidial concentration in each formulation was adjusted at 2×10^9 CFU/m^2.

2.6. Field trials

The isolate with the highest virulence *in vitro* against *R. microplus* was now tested in the field. In addition to a control treatment, two aqueous (water and

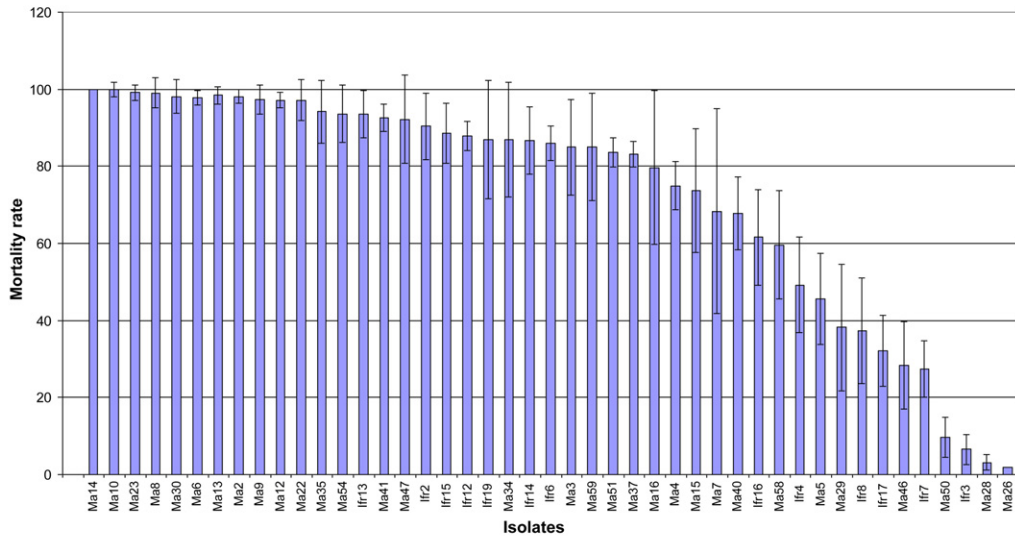

Fig. 1. Mortality of *R. microplus* larvae caused by *M. anisopliae* (Ma) and *I. fumosorosea* (Ifr). Columns represent means ± SEM ($n = 4$). Isolates Ifr 1, Ifr 5, Ifr 9, Ifr 10, Ifr 11, Ifr 18, Ifr 20, and Ifr 21 were not included for their inability to infect *R. microplus* larvae.

Citroline) and two solid (Celite and wheat bran) formulations of the fungal isolate were tested (Table 2).

All aqueous and solid formulations were adjusted at 2×10^9 CFU/m^2: aqueous formulations were dissolved in water at 200 L/ha during aspersion in the field; solid formulations were adjusted to a dose of 50 kg/ha. Aqueous and solid formulations were dispensed with a power sprayer (EFCOR, Model IS2026). The experimental field was located at rancho "El Peregrino" (19°12′25″N; 103°43′21″W) of Universidad de Colima. This area has several hectares covered with pasture beds of *Cynodon plectostachyus* (L.), naturally infested with *R. microplus*. A preliminary survey of the tick population indicated that these were abundant and with a highly contagious distribution. Two field experiments were conducted.

2.6.1. First field trial

An area of 1.85 ha, was divided in 5 irregular plots with an area ranging from 3645 to 3725 m^2. Each plot was used to apply one of the five treatments described above. The pre-treatment tick population in the field plots was estimated as follows: a 0.5 m^2 (1 m × 0.5 m) fabric was attached to the leg of a person that walked through the pasture bed; a 6 m diameter circle was traversed in a spiral path until the center was reached. The fabric was transported in a plastic bag to the laboratory where ticks were counted (Cornet et al., 1984; Pegram et al., 1993; Fernández-Ruvalcaba, 1996). Five samples per experimental plot were taken to assess the tick population. Twenty-four hours after tick counting plots were randomly selected to receive one of the treatments. Tick counting was repeated 14 days after treatment application. The efficacy of the each formulation was estimated as the percentage of tick reduction attributable to the treatment when compared to the control group (Morin et al., 1996; Bittencourt et al., 2003) using:

$$\%\,\text{efficacy} = \frac{(\text{mean tick no. control group}) - (\text{mean tick no. treated group})}{\text{mean tick no. control group}} \times 100$$

2.6.2. Second field trial

The natural larval population was eliminated within a 324 m^2 plot by applying permethrin (Ambush 50, Syngenta Agro S. A. de C. V.). Tick absence was corroborated 7 days after pesticide application (Tsicritsis and Newland, 1983; Awasthi and Anand, 1984; Imgrund, 2003). The area was divided in 25 experimental units. Each experimental unit was a 3 m × 3 m plot with 1 m separation on all sides from other plots. Separating areas were accomplished by removing the grass and partially paving the area with powdered lime; these "corridors" were used to access each plot and to prevent tick dispersion among treatment plots. About 20,000 *R. microplus* larvae were introduced at the center of each experimental unit. Treatments were applied ∼48 h after tick introduction during late afternoon. Aqueous formulations (Control, Tween, Citroline and Celite) were dispensed with a power sprayer (STIHL, Model SR 420, Andreas Stihl AG & Co., Wailblingen, Germany), while a wheat bran formulation was dispensed by hand. In total, the experiment had five treatments with five replicates each in a Latin square design. Tick counts started 14 days after treatment and were repeated every 7 days until day 35; cloth dragging was chosen as the sampling procedure (Solberg et al., 1992; Bittencourt et al., 2003). The efficacy of the each formulation was estimated as in the first field trial. Temperature and humidity records were taken with a data logger (HOBO, Onset, CO, USA) throughout the experiment.

ANOVA was used to test for differences in the number of larvae that survived at the end of the experiments. The Tukey test was used to separate treatment means when $p < 0.05$ (Sokal and Rohlf, 1981). All analyses were con-

Table 3
Concentration–mortality relationship (CL_{50}) in *R. microplus* larvae of *M. anisopliae* isolates.

Isolates	>LC_{50}[a]	>95% FL[b]
Ma 14	1.1×10^4a	5.5×10^3 to 2.4×10^4
Ma 13	1.9×10^4a	1.0×10^4 to 3.3×10^4
Ma 12	2.3×10^4a	>7.4×10^3 to 5.6×10^4
Ma 41	9.2×10^4a	5.4×10^4 to 1.5×10^5
>Ma 9	1.0×10^5a	>5.3×10^4 to 2.0×10^5
>Ma 8	1.6×10^5a	>6.2×10^4 to 2.4×10^5
Ma 35	1.7×10^5a	6.4×10^4 to 4.5×10^5
>Ma 6	>2.9×10^5a	>1.6×10^5 to 5.2×10^5
>Ma 2	6.4×10^5a	>4.4×10^5 to 9.5×10^5
Ma 30	7.1×10^5a	3.1×10^5 to 1.9×10^6
Ma 10	9.3×10^5a	6.1×10^5 to 1.4×10^6
Ma 47	2.4×10^6b	1.5×10^6 to 3.6×10^6
Ma 23	2.7×10^6b	5.3×10^5 to 1.4×10^7
Ma 22	5.2×10^6b	3.3×10^6 to 8.2×10^6
Ma 49	5.9×10^6b	3.1×10^6 to 1.2×10^7

Different letters within the LC_{50} column indicate statistical differences.
[a] LC_{50} (conidia/mL).
[b] Fiducial limits (95%).

ducted using the SAS statistical software (SAS Institute, 1997).

3. Results

3.1. Pathogenicity assays

All isolates of *M. anisopliae* were pathogenic to *R. microplus* at the 10^8 conidia/mL concentration. The lethal activity ranged from 2 to 100% larval mortality and 15 of the 33 isolates caused mortality values between 93 and 100%. Isolates of *I. fumosorosea*, caused from 5 to 94% larval mortality. Among *I. fumosorosea* isolates, Pfr 13 caused the highest mortality rate; in contrast, isolates Pfr 1, Pfr 5, Pfr 9, Pfr 10, Pfr 11, Pfr 18, Pfr 20, and Pfr 21 did not infect *R. microplus* larvae (Fig. 1).

3.2. Virulence assay

The LC_{50} values were estimated for the fifteen most virulent isolates of *M. anisopliae* and ranged between 1.1×10^4 and 5.9×10^6 conidia/mL. According to the 95% FL, eleven of these were the most virulent isolates with LC_{50} values ranging between 1.1×10^4 and 9.3×10^5 conidia/mL. In contrast, the isolates Ma 47, Ma 23, Ma 22, and Ma 49 had the lowest virulence, with LC_{50} values that ranged between 2.4×10^6 and 5.9×10^6 conidia/mL (Table 3). Virulence for Pfr 13 was not estimated due to a lack of relationship between conidial concentration and larval mortality. The LC_{50} values were not distinguishable for several *M. anisopliae* isolates with high virulence against *R. microplus*. Therefore, one of the isolates that appeared to have the highest virulence, Ma 14, was selected to be used in the field trials.

3.3. First field trial

Two weeks after the first application there was a reduction in the larval tick population which ranged from 0 to 94.2%. The treatment with the highest reduction on tick numbers corresponded to the solid formulation (Table 4).

3.4. Second field trial

Two weeks after the beginning of the experiment, the wheat bran treatment was the single formulation that caused significant larval tick reduction (54.9% efficacy). However, within the following sampling dates, there were no differences between treatments, and the following trends were observed: (1) after 21 days, the Tween and wheat bran treatments promoted larval reduction (47.8 and 58.3%, respectively) and (2) the Citroline treatment showed effects only 28 days after application, with lower efficacy (32.8%) than the wheat bran and Tween treatments (54.2 and 61.1%, respectively) (Table 5). The Celite treatment showed no effect throughout the experiment.

During experiments, the maximum, mean, and minimum temperature recorded was 30.71, 24.84, and 19.42 °C, respectively, and the maximum, mean and minimum relative humidity recorded was 78.6, 53.2, and 32.0%, respectively.

4. Discussion

Entomopathogenic fungi are able to infect and cause high mortality rates in various species and stages of ticks *in vitro* (Monteiro et al., 1998a,b,c; Gindin et al., 2001; Samish et al., 2001, 2004a,b; Polar et al., 2005a,b,c). Our results indicated that slightly over 30% of the isolates of *M. anisopliae* tested were highly pathogenic against *R. microplus*, suggesting an enormous potential for this fungal species to be used for tick control. Moreover, while the LC_{50} values were variable, most of them had a high virulence range, and four of them had values within 1.1×10^4 and 9.2×10^4 conidia/mL. These LC_{50} values are practical for the development of a myco-acaricide aimed to control ticks in integrated management programs. Although this study tested only the Ma 14 isolate in the field, the other isolates may also be efficient to control *R. microplus* and other tick species; however, further tests are needed under field conditions.

Table 4
Number of *R. microplus* larvae 14 days after sprays of *M. anisopliae* (isolate Ma 14) conidia formulations (2×10^{13} conidia/ha) on *C. plectostachyus* pasture beds (first field trial).

Treatements	Mean initial number of *R. microplus* larvae (means ± SEM)	Larval density (means ± SEM)	% reduction
Control	1400 ± 774.5	606.5 ± 355.5	56.7
Tween	2252 ± 651.2	1421 ± 213.3	36.9
Citroline	2257 ± 480.1	2615 ± 386.1	0
Celite	3167 ± 705.2	1021 ± 420.5	67.8
Wheat bran	1802 ± 651.7	105.5 ± 11.5	94.2

Table 5
Number of *R. microplus* larvae at various intervals (days) after aspersion of *M. anisopliae* (isolate Ma 14) conidia formulations (2×10^{13} conidia/ha) and their efficacy on *C. plectostachyus* pasture beds (second field trial).

Treatments	14[a]	Efficacy (%)	21	Efficacy (%)	28	Efficacy (%)	35	Efficacy (%)
Control	762.2 ± 835.8 ab		604.2 ± 631.6 a		156.8 ± 108.9 a		11.8 ± 9.3 a	
Tween	1007.4 ± 1393.2 ab	0	315.4 ± 447.4 a	47.8	61 ± 74.4 a	61.1	11.6 ± 10.1 a	0
Citroline	1630.6 ± 1310.7 a	0	830.4 ± 897.9 a	0	105.4 ± 37.6 a	32.8	13.2 ± 8.7 a	0
Celite	1680.4 ± 1206 a	0	1346 ± 952.9 a	0	262.4 ± 275.1 a	0	16.4 ± 11.5 a	0
Wheat bran	343.8 ± 253.5 b	54.9	252.2 ± 150.8 a	58.3	71.4 ± 47.6 a	54.2	8.4 ± 4.1 a	28.8

[a] Different subscripts within a column indicate values statistically different; all analyses on data after root transformation.

Remarkably, all isolates completed their life cycles and formed conidiophores on *R. microplus* larvae *in vitro*; this trait is important, since a new generation of conidia growing on dead larvae ensures the persistence of infectious inoculums in the tick micro-environment, this new source of inoculum has potential of transmission to other ticks (Gindin et al., 2001).

In the present study the fungal isolates were not isolated from arachnids. Our results indicate that isolates with high pathogenicity against ticks could be found from samples derived from soil on the last instars of the great wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). This property is presumably advantageous in the development of a myco-acaricide; this agrees with the findings of Monteiro et al. (1998a,b) who reported that an ant derived isolate was more effective than a tick derived isolate. However, according to Goettel et al. (1990) a fungal isolate may be more pathogenic against the host from which it was obtained than to other novel hosts; it has also been suggested that the most pathogenic fungal isolates against a particular host are found within the same host habitat.

The field trials with the *M. anisopliae* isolate Ma 14 indicated that *R. microplus* can be effectively controlled using different conidia formulations. However, it was also clear that the carrier used in a given formula may have impact on the efficacy of a given isolate. Samish et al. (2004a,b) recently discussed this problem on field spore aspersion highlighting the need for additional research on the interaction between the environment, host, carrier and spores. In the present study, the addition of water to the Celite containing treatment presumably hindered the action of *M. anisopliae* spores against tick larvae. Also, oils have been proposed as useful carriers to suspend conidia for the control of insect pests (Prior et al., 1988; Lomer et al., 1992; Bateman et al., 1993) and tick control (Maranga et al., 2005). A Citroline-conidia formulation of *M. anisopliae* has been successfully used in Mexico for the control of the Central American locust, *Schistocerca piceifrons* Walker (Orthoptera: Acrididae), promoting up to 99% decline in field bands, 13 days after field application (Hernández-Velázquez et al., 2003). However, in the present study the Citroline formulation did not promote a significant reduction of tick larvae numbers; this could be attributed to several factors. In particular, Citroline was used in combination with other carriers including water, as opposed to the formulae used by Hernández-Velázquez et al. (2003) where Citroline was used as the sole component and applied as Ultra Low Volume (ULV). However, there is evidence suggesting that the grass species grown in a pasture bed along with weather conditions also affect the action of *M. anisopliae* to control *R. microplus* (Kaaya and Hassan, 2000; Basso et al., 2005).

It was surprising that the wheat bran formulation was highly effective both dispensed by hand or with a mechanical dispenser. Pilot *in vitro* studies indicated that *M. anisopliae* completed its full cycle on wheat bran particles within 5 days, producing conidia in large numbers (Lezama-Gutiérrez and Ángel-Sahagún, unpublished observations). Presumably the use of wheat bran as a carrier served to amplify the conidia on the field, improving the capacity of *M. anisopliae* as a myco-pesticide for tick control. Additional research is needed to test other grain derived carriers in the formulation of myco-acaricides.

Serial application of myco-acaricides over a time period may be a promising strategy for tick control. In fact, the use of *M. anisopliae* and *B. bassiana* conidia once a month over a 6-month period promoted a significant reduction of *Rhipicephalus appendiculatus* populations in western Kenya (Kaaya and Hassan, 2000). In this study, within 2 weeks, the Celite and wheat bran treatments decreased the tick population up to 67.8 and 94.1%, respectively on pastures naturally infested larvae; however, a tendency to naturally reduce the tick population was observed in all treatments (Tables 4 and 5). Therefore, pasture application of conidia could be a promising strategy when formulations and environmental conditions support fungal development and increase the probability of epizootics (Polar et al., 2008).

The optimal temperature for the majority of the isolates of *Metarhizium* spp., studied by Ouedraogo et al. (1997) was 25 °C; the temperature in our field conditions ranged from 19.4 to 30.7 °C, with a similar mean temperature (24.8 °C) to the temperature reported by the authors mentioned above; we consider that the temperature did not affect the action of the entomopathogenic fungi in the formulations against tick larvae under our field conditions. The other important environmental factor that determines the effectiveness of the infection of entomopathogenic fungi is the relative humidity. Usually, the spores germinate under high humidity (Ferron, 1975); however, Müller-Kügler (1965) reported that the insect infection is possible to achieve at low HR (40–50%), and stated that the humidity in the cuticle plays an important role in the fungal infection. Recently, Michalaki et al. (2006) reported more effectiveness in *M. anisopliae* at 55% than 75% of relative humidity. In our study the relative humidity data had a range between 32.0 and 78.6%, and a mean relative humidity of 53.2%. We sustain that HR also did not affect the action of our formulations against tick larvae under our field conditions, based on the scientific evidences mentioned previously; however, the fungal sporulation demands high relative humidity on the

cadaver that could affect the potential for future epizootics (Bajan and Karnikova, 1968).

In conclusion, our results suggest that the conidial formulation of *M. anisopliae* with wheat bran was the most effective to reduce the larval population of *R. microplus* in the field. This formulation could be implemented in the future as part of an IPM strategy to control cattle ticks. Further research conducted with solid formulations involving other grain fibers may render useful information to develop fungi carriers that might enhance tick control.

Acknowledgements

This research was partially supported by SAGARPA-CONACYT (Mexico's Secretariat of Agriculture, Livestock Production, Rural Development, Fishery and Food – The National Science and Technology Council) – 2004-C01-128/A-1 project and by Universidad de Colima – Facultad de Ciencias Biológicas y Agropecuarias, and Facultad de Medicina Veterinaria y Zootecnia, Tecomán, Colima, México.

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