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OXFORD

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Abstract

The effect of the fungus *Metarhizium anisopliae* Ma14 strain, D-limonene, and cypermethrin, alone and combined, on the mortality of *Rhipicephalus sanguineus* Latreille larvae was evaluated. Eight separate groups with 25 tick larvae were inoculated with the fungus, cypermethrin, and D-limonene, and four groups were used as untreated controls. The groups were inoculated with serial dilutions of each treatment material: for example, conidial concentrations were 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8. A complete randomized experimental design was used. Significant differences were obtained between fungal concentrations, with larval mortalities ranging from 29 to 100%; the D-limonene concentrations showed significant differences, with mortalities that ranged from 47.9 to 82.6%, and cypermethrin mortalities ranged from 69.9 to 89.9% when each was applied alone. In the combined application, the serial dilution of the Ma14 fungus plus cypermethrin at 0.1% concentration caused mortalities ranging from 92.9 to 100%; the mix of serially diluted Ma14 plus D-limonene at 0.1% caused mortalities from 10.3 to 100%; and the mix consisting of serially diluted D-limonene plus cypermethrin at 0.1% caused mortalities from 7.4 to 35.9%. Further laboratory and field research could show that these materials, alone and in combinations, are useful in future tick management and control programs.

Key words: biological control, chemical control, tick mortality, brown dog tick, entomopathogenic fungus

Brown dog tick, *Rhipicephalus sanguineus* Latreille, is the most widespread tick in the world. All life stages prefer warmer climates. They attack dogs but can occasionally parasitize other hosts, including humans and other mammals (Dantas-Torres 2010). High levels of infestation of brown dog tick can cause skin irritation and damage in dogs, and the population can reach pest proportions in houses and kennels. *Rhipicephalus sanguineus* can vector disease organisms of zoonotic concern in dogs—canine ehrlichiosis (*Ehrlichia canis*)

(Fourie et al. 2013), canine babesiosis (*Babesia canis*) (Lord 2014), *Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia rickettsia* (Dantas-Torres 2008).

Tick control is based primarily on chemical treatment. Synthetic acaricides, tested against the brown dog tick, are applied by immersion, spraying or bathing, and subcutaneous injections (Ayodhya 2014). Cypermethrin, a systemic pyrethroid insecticide with low toxicity for mammals, is commonly used with domestic animals

(Kumar et al. 2008). However, chemical control has potentially high ecological costs, including damage to nontarget organisms, and ticks are also likely to develop pesticide resistance (Miller et al. 2001). Alternative methods or strategies of control of brown dog tick are required (Taylor 2001).

Biological control has proven to be a feasible alternative to reduce tick populations; the entomopathogenic fungi provide promise (Garcia et al. 2004). Some studies have emphasized the role of the entomopathogenic fungi such as *Cordyceps ≈ Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin that cause egg and larval mortality in *R. sanguineus* (Monteiro 1998a,b; Garcia et al. 2008), and *Verticillium lecanii* against *Ixodes scapularis* (Zhioua et al. 1999).

Botanical insecticides, under the umbrella of bio-rational control, are also promising alternatives. Botanical insecticides can reduce host arthropod populations as feeding deterrents, repelling the ectoparasites, or by other modes (O'Farril, 1995). D-limonene (1-methyl-4-isopropenyl-1-cyclohexene), found in citrus, is often extracted with either pressure or steam from the peel of these fruits. The botanical molecule is generally recognized as safe by the U.S. Food and Drugs Administration and the U. S. Environmental Protection Agency. D-limonene's mode of action is the dissolving of the protective layer of wax from the exoskeletons of the arthropods, causing them to suffocate and die (Direct Chem 2001). D-limonene is an active ingredient in several products such as flea dips for dogs and cats and pesticides for indoor pest control (Trumble 2002).

Sometimes the application of a single strategy or tactic—chemical, bio-rational, biological, cultural, or other means—is enough to control a targeted organism. But in other situations it is necessary to incorporate or combine more tactics to reduce the population of concern.

Our objective was to determine the effect of the entomopathogenic fungus *M. anisopliae*, cypermethrin, and D-limonene, applied alone or in combination, on the larval mortality of *R. sanguineus* under laboratory conditions.

Materials and Methods

The study was conducted in the Laboratory of Biological Control of the Universidad de Colima (LBC-UdeC) in the Tecomán Colima, Mexico.

Collection and Preparation of Adults of Brown Dog Tick
Adult engorged ticks were manually collected from naturally infested dogs in the urban zone of Tecomán, Colima. Ticks were deposited in plastic containers of 500 ml and then covered with a perforated lid. They were transported to the laboratory, disinfected with 0.1% sodium hypochlorite for 10 s, and then rinsed three times with distilled water to remove the hypochlorite. Ticks were then placed in Petri plates 90×100 mm containing a double layer of filter paper (Whatman No. 1; Whatman International Ltd, England) moistened with distilled water and incubated at $25 \pm 1\,°C$, 90% RH, and a photoperiod of 12:12 (L:D) h (Benjamin et al. 2002) until ready for egg laying (Kaaya and Hassan 2000). Eggs were incubated under the same conditions as adults until the eclosion of the larvae. Larvae were allowed to rest for 7 d, under the same conditions, before use in the bioassays (Gidin et al. 2002).

Preparation of Entomopathogenic Fungus, Cypermethrin, and D-Limonene
Metarhizium anisopliae Ma14 strain was selected based in its proven pathogenicity against other tick species. It is deposited in the Entomopathogenic fungi collection of the LBC-UdeC. The fungus was grown on Sabouraud dextrose agar, enriched with yeast extract (1%) and 500 ppm of the antibiotic chloramphenicol; it was incubated for a period of 21 d at $25 \pm 1\,°C$ and a photoperiod of 12:12 (L:D) h. For mass production, the fungus was grown on rice grains (Prior and Jollands 1988). Once the conidia were obtained, they were dried for 8 d in a hood at $25\,°C$ and 53% RH, and then stored at $5\,°C$ until used in the laboratory. Conidial concentrations were determined with an improved Neubauer hemocytometer (Reichert Scientific Instruments, USA) based in an aqueous solution. Eight concentrations were adjusted by dilution from 1×10^8 in series down to 1×10^1 conidia/ml (Arthurs and Thomas 2001) with a 0 conidia/ml control.

Cypermethrin, a commonly used pesticide to control *R. sanguineus* (Alcaíno et al. 1995), was tested in doses selected according to manufacturer recommendation (1 liter of the commercial product per 1,000 liter of water = 0.1% of final formulation; Prontuario de Especialidades Veterinarias 2007). Final formulation was then diluted to concentrations of 1×10^{-1} to 1×10^{-8}%. A 0 concentration control was also used.

An oil solution of the terpene D-limonene was used to obtain diminishing concentrations from 10, 1, 1×10^{-1} down to 1×10^{-6}%. A 0 concentration control was also used.

Bioassay Procedure
Groups of 25 17-d-old tick larvae were formed; they were adhered to masking tape to facilitate their handling. Tick larval inoculation was done by immersion technique for 5 s (Kaaya et al. 1993); distilled water containing 0.1% Tween 80 was used for all treatments. Thirty-six groups of 25 larvae were split into four subsamples and inoculated with the eight serial dilutions of *M. anisopliae* and one control; 40 groups were treated with cypermethrin and 36 with D-limonene were applied in the same manner (Table 1). Untreated controls for each treatment were immersed in distilled water added with Tween 80 at 0.1%. Ticks were then held under the same conditions as indicated above. Treatments were distributed in a completely randomized design with four replications per treatment. Data transformation was performed using Y =arc sin √p, before the ANOVA, and means were separated using Tukey's test ($P = 0.05$; SAS Institute Inc 1997).

Evaluations were also done of the effect of mixing the fungus with D-limonene and the cypermethrin, as well as the D-limonene mixed with cypermethrin. Concentrations of the fungus, the same as above, were used and blended with D-limonene at 1% and cypermethrin at 0.1%. The blends of D-limonene, using the same serial dilutions mentioned previously, were mixed with cypermethrin at 0.1%. All tests followed the immersion technique. A total of 54 groups (two subsamples for each treatment combination) of 25 larvae were inoculated for each replicate. Each treatment combination included an untreated control and was replicated four times. Ticks were examined microscopically for fungal disease; mortality was recorded daily for 7 d (Zhioua et al. 1997).

Results

Significant differences were obtained in the percentage of tick larval mortality caused by different concentrations of *M. anisopliae* ($F = 25.9$, $P = 0.0001$), D-limonene ($F = 11.77$, $P = 0.0001$), and cypermethrin ($F = 17.99$, $P = 0.0001$) applied as individual treatments (Table 1). Larval mortality caused by Ma14 ranged from 29.1% to 100%. Mortality for all fungal treatments differed from the control with the exception of the 1×10^1 concentration (Table 1).

Table 1. Average percentage of mortality of larvae of *R. sanguineus* caused by different concentrations of the fungus *M. anisopliae* (Ma14 strain), percent D-limonene, and percent cypermethrin

M. anisopliae (Ma 14)	Mortality (%)	Terpene (D-Limonene)	Mortality (%)	Acaricide (Cypermethrin)	Mortality (%)
Control	3.0b	Control	7.2c	Control	2b
1×10^1	29.1b	0.000001	69.2ab	0.00000001	83.8a
1×10^2	83.2a	0.00001	47.9b	0.0000001	89.9a
1×10^3	89.9a	0.0001	82.6a	0.000001	82.1a
1×10^4	96.8a	0.001	79.07ab	0.00001	79.8a
1×10^5	94.1a	0.01	72.8ab	0.0001	78.9a
1×10^6	92.5a	0.1	62.1ab	0.001	86.9a
1×10^7	98.8a	1	80.9ab	0.01	85.1a
1×10^8	100a	10	75.8ab	0.1	60.8a
				1.0	77.2a
F value	25.9	*F*. Cal.	11.77	*F*. Cal.	17.99
Pr >F	0.0001	*Pr >F*	0.0001	*Pr >F*	0.0001
CV%	17.5	CV%	18.2	CV%	14.84

Means within a column followed by the same letter are not significantly different (Tukey test $P < 0.05$).
CV%, coefficient of variance.

Table 2. Percentage mortality of larvae of *R. sanguineus* caused by different mixtures and concentrations of the fungus *M. anisopliae* (Ma14 strain), D-limonene, and cypermethrin

M. anisopliae Ma 14 + Cypermethrin (0.1%)	Mortality (%)	*M. anisopliae* Ma 14 + D-Limonene(1%)	Mortality (%)	D-Limonene + Cypermethrin (0.1%)	Mortality (%)
Control	4b	Control	3d	Control	6a
1×10^1	92.9a	1×10^1	29.4bc	0.000001	23.8a
1×10^2	93.1a	1×10^2	14.7bcd	0.00001	11.6a
1×10^3	98.9a	1×10^3	10.3cd	0.0001	33.3a
1×10^4	98.9a	1×10^4	23.5b	0.001	10.2a
1×10^5	98.8a	1×10^5	91.3a	0.01	20.2a
1×10^6	100a	1×10^6	98.9a	0.1	7.4a
1×10^7	93.3a	1×10^7	100a	1	16.3a
1×10^8	100a	1×10^8	100a	10	35.9a
F. Cal.	28.51	*F*. Cal.	92.41	*F*. Cal.	1.59
Pr >F	0.0001	*Pr >F*	0.0001	*Pr >F*	0.1744
CV%	12.76	CV%	13.93	CV%	52.03

Means within a column followed by the same letter are not significantly different (Tukey test $P < 0.05$).
CV%, coefficient of variance.

D-limonene caused tick mortalities ranging from 47.9% to 82.6% (Table 1). Highest larval mortality (82.6%) at the concentration of 0.0001 did not differ from several other concentrations, 0.000001 to 10 (mortality ranged from 62.1% to 82.6%); all treatment concentrations had mortality different than the control (Table 1).

Mortality levels were not significantly different at any of the cypermethrin concentrations, ranging between 60.8% and 89.9% (0.1% and 0.0000001%, respectively; Table 1). Mortality at all cypermethrin concentrations was different than the control (Table 1).

Significant differences in tick mortality were caused by the effect of different concentrations of Ma14 + cypermethrin 0.1% ($F = 28.51$, $P = 0.0001$) and Ma14 + D-limonene 1% ($F = 92.41$, $P = 0.0001$; Table 2). However, significant differences, including from the control, were not obtained for the concentrations of D-limonene + cypermethrin 0.1% ($F = 1.59$, $P = 0.1744$; Table 2). The treatment combinations of different concentrations of Ma14 and cypermethrin 0.1% were not statistically different from each other, with a range of mortality from 92.9% to 100%; all treatment combinations were different from the control (Table 2). The concentrations of Ma14 + D-limonene 1% showed the highest larval mortalities with the concentrations of Ma14 from 1×10^5 to 1×10^8 conidia (ranging from 91.3% to 100%, respectively; Table 2).

Discussion

Metarhizium anisopliae is able to infect and kill larvae of *R. sanguineus*, causing tick mortalities up to 100% 9 d postinoculation and using the concentration of 1×10^8 conidia/ml (Garcia et al. 2008). Our results with *M. anisopliae* support earlier reports on the impact of the fungus on *R. sanguineus*, and *Ixodes scapularis* (Benjamin et al. 2002). The role of spore density has been reported to play an important role in reaching the threshold for an effective penetration of the tick's cuticle and subsequent death of the larva (Zhioua et al. 1997). Reis-Menini et al. (2008) evaluated formulations of *M. anisopliae* and *B. bassiana* against *R. sanguineus* females and reported that their formulations improved the adhesion of the fungus on the cuticle in comparison with the aqueous solution. We evaluated different concentrations of the *M. anisopliae* fungus in aqueous

solution containing Tween 80 at 0.1%, breaking the surface tension of the conidia and possibly improving the adhesion to tick cuticle. A synergistic effect was observed with the treatments of *M. anisopliae* combined with cypermethrin at 0.1%, causing >93% mortality in comparison with the separate treatments; similar results were reported by Hornbostel et al. (2005) with the fungus and permethrin against *I. scapularis*.

When the fungal treatments were mixed with D-limonene at 1%, mortality was not improved compared to either treatment used alone; mortality for the mixture did reach >90% at 1×10^5 conidia/ml and approached 100% at the three higher conidia levels. This effect was previously reported by Bahiense et al. (2006) using *M. anisopliae* and deltamethrin applied against *Boophilus microplus* where 36.5% mortality was seen with concentrations ranging between 0.39 and 6.25 ppm of deltamethrin applied alone; mortalities of 16.5%, 55%, and 96.9% with the concentrations of 2.2×10^5, 2.2×10^6, 2.2×10^7 up to 2.2×10^8 conidia/ml of the fungus applied alone, respectively; and with mixed treatments causing 68%, 54%, 100%, and 100% using the highest concentration of deltamethrin with the fungal concentrations, respectively. It was expected that concentrations of D-limonene mixed with the fungal concentrations would improve the suspension of the conidia, producing an oily emulsion and increasing the spore density that facilitated the adhesion of the conidia to the larval cuticle and thereby causing higher mortality (Bateman et al. 1993). Oily plant extracts have an acaricide effect against ticks (Abdel-Shafy and Soliman 2004); the oily plant extracts of plants belonging to the families Meliaceae, Lamiaceae, Labiatidae, and others, have acaricidal effects against ticks due to their chemical composition. Tick larval mortalities in this study using D-limonene were likely caused by the physical nature and chemical composition of the molecule.

Antagonism was detected in the treatments constituted by cypermethrin 0.1% with different concentrations of D-limonene. For unknown reasons they did not reach >36% mortality.

Combinations of *M. anisopliae*, D-limonene, and cypermethrin caused tick mortalities that often reached 100%. Several concentrations of *M. anisopliae* with D-limonene 1.0% or cypermethrin 0.1% produced mortality that was consistently around 100%. Further research could show that these materials, alone and in combinations, are useful in future tick management and control programs.

Acknowledgments

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