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In vitro Efficacy Testing of a Commercial Formulation of the Acaropathogenic Fungus *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) strain F52 against the southern cattle fever tick *Boophilus microplus* Canestrini (Acari: Ixodidae).

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ABSTRACT

A commercially available formulation of *Metarhizium brunneum* strain F52, Met52®EC, was tested *in vitro* against the off-host stages of the southern cattle fever tick, *Boophilus microplus*. At the highest test concentrations all engorged adult female ticks became infected after exposure with LD-50 at day 6 post-inoculation. However, much oviposition ensued prior to the death of the ticks. At the highest dose, 1 x 10^8 cfu/ml, the weight of the egg mass was reduced by approximately 80%. Viability of the eggs was not affected by the treatment. Mortality of the larval stage was dose-dependent with nearly 100% at the highest dosage tested, and around 93% at the recommended application rate.

Additional Index Words: Livestock-pests, biocontrol, pasture-treatment.

Ticks and tick-borne diseases affect animal health and impede the development of livestock production systems where they occur (de la Fuente et al. 2008, Brites-Neto et al. 2015). The southern cattle fever tick, *Boophilus microplus* Canestrini, is arguably the most economically important ectoparasite of livestock worldwide (Jongejan and Uilenberg 2004, Grisi et al. 2014). Productivity in infested cattle is directly affected by the blood-feeding habit of the southern cattle fever tick, and indirectly by its role as vector of the microbes that cause bovine babesiosis and anaplasmosis (Pérez de León et al. 2010). Bovine babesiosis caused by *Babesia bovis* or *B. bigemina* is considered the most significant cause of morbidity and mortality in cattle raised in tropical and subtropical regions of the world where *B. microplus* is established (Pérez de León et al. 2014). Drugs and vaccines have been used to mitigate the impact of bovine babesiosis. However, a more widely adopted practice involves the use of acaricides to manage *B. microplus* infestations and reduce the risk of bovine babesiosis and anaplasmosis transmission to cattle (Guerrero et al. 2014).

The southern cattle fever tick ranks sixth among the most detrimental arthropods to agriculture that are resistant to pesticides (Whalon et al. 2008). Reliance on chemical controls has inevitably led to acaricide resistance in several *B. microplus* populations around the world (Rodriguez-Vivas et al. 2014, Lopez-Arias et al. 2015). Of major concern is the development of tick populations resistant to multiple classes of commercially available acaricides (Miller et al. 2013, Klafke et al. 2017). New control technologies are needed in parts of the world where multiple acaricide resistance in *B. microplus* threatens the productivity and profitability of livestock production.

Mycoacaracides represent a non-chemical alternative for *B. microplus* control (Fernandes et al. 2011, Beys-da-Silva et al. 2012). The fungus *Metarhizium brunneum* Petch is one of the acaropathogenic species that has been investigated for that purpose (Fernandes et al. 2012), although much of the literature on this fungus strain referred to *Metarhizium anisopliae* F52 (Bharadwaj and Stafford 2012, Behle et al. 2013). High tick mortality caused by several *M. brunneum* isolates have been documented through *in vitro* testing (Guedes-Frazzon et al. 2000, Leemon & Jonnson

2008, Lubeck et al. 2008). However, the susceptibility to *M. brunneum in vivo* can vary depending on the tick population, environmental conditions, and the fungal preparation (Bahiense et al. 2006, Webster et al. 2015, 2016). Thus, trials against on-host adult ticks by application to infested cattle has given inconsistent results. Barcelos-Correia et al. (1998) found no reduction in numbers of ticks greater than 4 mm length after treatment of stabled cattle in Brazil. Although Alonzo-Diaz et al. (2007) reported >40% control after repeated treatments in naturally infested cattle in Mexico, this level of reduction could be considered marginally economical even as part of an integrated control program.

Advances in formulation chemistry have enabled the commercialization of *M. brunneum* products against ticks, including *B. microplus* (Alves et al. 2003, Camargo et al. 2012), with carriers that enhance shelf-life in terms of conidial viability (Bharadwaj and Stafford 2010, Camargo et al. 2016). However, the efficacy against *B. microplus* of a formulation of the F52 strain under the commercial name Met52®EC remained undetermined. Herein we report the results of in vitro tests to determine efficacy against adult and larval stages of *B. microplus*. The findings are discussed in the context of integrated efforts to manage *B. microplus* populations.

MATERIALS AND METHODS

Tick Rearing. All experiments were conducted at the United States Department of Agriculture-Agriculture Research Service (USDA-ARS) Cattle Fever Tick Research Laboratory located at Moore Air Base near Edinburg, Texas, and all procedures involving the use of cattle for tick rearing were approved by the Institutional Animal Care and Use Committee. Fever ticks were reared using methods described in detail by Davey et al. (1982). Briefly, stanchioned calves were infested with larvae that completed development in approximately three wks at which time engorged females dropped from the host-animal. The engorged females were gathered and used within 24 hr of their dropping off the host-animal for the experimental treatments, or maintained under colony conditions for egg laying. Masses of eggs laid by the female ticks in the laboratory were placed in cotton stoppered vials for larval hatch. Larvae emerging from these eggs were held under colony conditions for two weeks before used for *in vitro* testing.

Metarhizium Preparations. The bioacaracide Met52®EC (Novozymes Biologicals, Salem VA) was purchased in 1L amounts as needed, and stored at 4°C until use. All tests were conducted with freshly opened containers. The product label indicates a formulation of 11% *M. brunneum* strain F52 as the a.i. = 5.5 x 10^9 cfu/g, with 89% petroleum distillate based carrier. Treatment dilutions were prepared starting with 0.36 ml of product added to 19.64 ml de-ionized water. This suspension served as the high concentration (1 x 10^8 cfu/ml). Ten-fold serial dilutions were prepared by adding 0.5 ml of each dilution to 4.5 ml de-ionized water giving mid- and low concentrations of 1 x 10^7 and 1 x 10^6 cfu/ml, respectively. The middle concentration equaled the manufacturers recommended application dose (MRD), and these concentrations approximated those found to be efficacious in previously published studies (Gindin et al. 2001, Ojeda-Chi et al. 2010).

Larval Immersion Test. Testing of tick larvae, 14d old unfed larvae (approx. 100 per replicate) were immersed for 30 sec at each of the three concentrations described above, then transferred to Petri dishes (10 cm diameter) containing 9.0 cm Grade 1 qualitative filter paper (Whatman), sealed with parafilm, and incubated at 25°C for seven days. The negative control (blank) treatment had larvae immersed in distilled water, and the positive control treatment was prepared by immersing larvae in the petroleum distillate carrier (10% liquid paraffin emulsifiable adjuvant oil) without the *Metarhizium*.

Adult Immersion Test. Replete females were collected as described above and used in treatments within 24 hr to ensure they were in pre-oviposition status. Ten replete females per replicate were immersed in each of the three dosage suspensions, or a distilled water control, for 30 sec. The ticks were then blotted dry and placed in a petri dish with 9.0 cm Grade 1 qualitative filter paper (Whatman) for 14 d at 25°C and 95% RH and sealed with parafilm. The ticks were affixed to a piece of double-sided tape (Fig. 1a and 1b). Mortality of ticks, judged by hyphal growth or discoloration, was scored daily. After 14 d, composite egg masses were collected and held in cotton stoppered glass vials for hatch. Percent hatch was scored on day 24 following the first day of observed larval eclosion.

Statistical Analyses. Treatment mortalities were corrected for control mortality using Abbott's formula prior to analysis (Abbott 1925). Means were compared pairwise by two-tailed t-test. Correlation was measured by least squares linear regression. Probabilities were calculated with the on-line program GraphPads <www.graphpads.com/quickcalcs>

RESULTS

The formulation of the *M. brunneum* F52 strain approved for commercialization in the U.S under the trade name Met52®EC, was active against *B. microplus* larvae and engorged females. Mortality was dose dependent. An effect on reproduction was ob-

served only in oviposition at the highest concentration tested.

Adult Immersion Tests. Maximum mortality (100%) was achieved at the highest concentration by day 12. Around 85% mortality was achieved with the low to medium doses by day 13 post-treatment. The 50% mortality rate was reached in six days at the high and medium treatment levels and in eight days at the low dose (Fig 2). Significant mortality to the adult stages resulted from exposure to Met52®EC, but death was often delayed until after oviposition had occurred

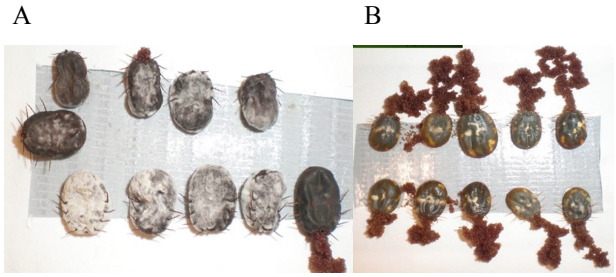

Fig. 1a, b. Engorged female fever ticks at 6 d post-inoculation (a) and controls (b) of same age showing hyphal growth from fungal infection and oviposition of egg masses.

Table 1. Adult immersion test results. Mean and standard deviation of egg mass weight (mg) at three treatment dosages and untreated control by replicate (n = 4 per mean) and pooled by treatment. Replicate means includes females that did not oviposit. Pooled data is for ovipositing females. Statistical comparison pairwise with two-tailed t-test.

Replicate	1	2	3	Pooled
Control	1.05 ± 0.22a	2.22 ± 0.15a	1.63 ± 0.32a	1.63 ± 0.54a
Low Dose	0.18 ± 0.15b	1.93 ± 0.11b	1.12 ± 0.34b	1.08 ± 0.77b
Mid Dose	0.04 ± 0.03b	2.08 ± 0.10a	1.09 ± 0.30b	1.17 ± 0.86b
High Dose	0.07 ± 0.06b	0.37 ± 0.11c	0.59 ± 0.28c	0.37 ± 0.27c

Means followed by the same letter in a column are not significantly different at p = 0.05

(Fig. 1a). Although a dosage effect on oviposition rate was noted, there was no significant difference (t = 0.91, df = 4, p = 0.41) in egg masses (Table 1) between the control (1.63 ± 0.54 mg), the low dose (1.08 ± 0.87 mg), and the MRD (1.07 ± 1.02 mg). However, at the highest concentration (1 x 10^8 cfu/ml) the egg mass (0.37 ± 0.27 mg) was reduced by 80% relative to the control (1.63 ± 0.54 mg), and this was significantly

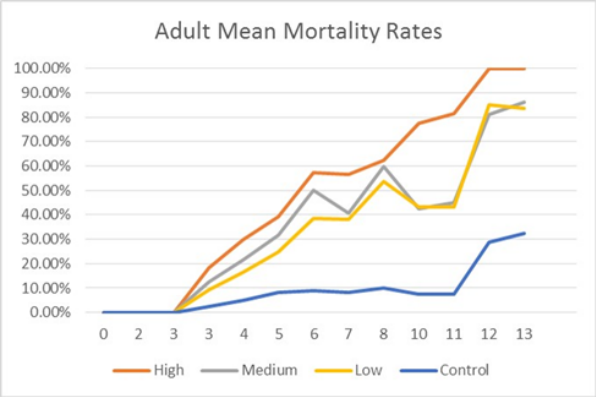

Fig. 2. Mortality curves over days post-inoculation for controls and three treatment dosages.

different (t = 4.56, df = 4, p = 0.02). There was no measurable effect on egg hatchability at any concentration (Fig 3) (t = 1.12, df = 4, p = 0.32).

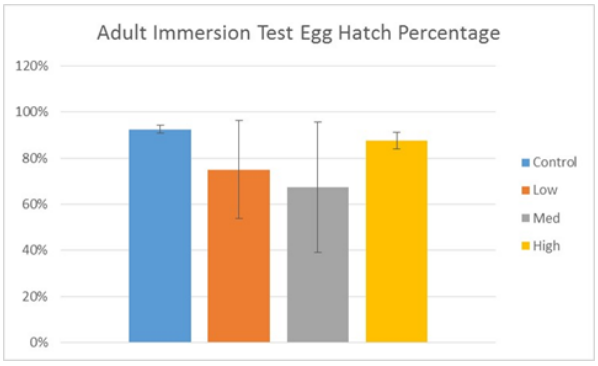

Fig. 3. Mean egg hatch (percent) at three treatment dosages and untreated control.

Larval Immersion Tests. For the controls, larvae were immersed in distilled water or in the diluted adjuvant without conidia. Control mortality with the adjuvant (6.2 ± 6.5%) was not significantly different from

Table 2. Larval immersion test results. Statistical comparison of mortality among control and treatments with pairwise, two-tailed, t-test.

Source	*t*-score	*df*	*p*
Control vs			
Low Dose	1.53	13	0.15
Mid Dose	19.7	13	0.0001
High Dose	37.9	12	0.0001
Mid Dose vs			
Low Dose	6.48	14	0.00001
High Dose	1.68	13	0.116

the water control mortality (4.65 ± 2.1%) (t = 0.74, df = 16, p = 0.47). Because it was relatively larger, the control mortality with the adjuvant was used for the Abbott's correction and for comparison to the treatments. Mortality was dosage dependent. With linear regression the coefficient of determination (r^2 = 0.55) was significant (F = 34.4, df = 1, 28, p = 0.0001).
The mean Abbott's corrected mortality at the low, medium and high doses were 22.7 ± 27.7, 93.3 ± 13.7, and 99.6 ± 11.2%, respectively. These were all significantly different from the controls (Table 2).

DISCUSSION

Our findings are the first report of efficacy against *B. microplus* for a formulation of *M. anisoplae* strain F52 that is commercially available in the U.S. However, that fungal strain has been reclassified and is specifically known as *M. brunneum* (Bharadwaj and Stafford 2012; Behle et al. 2013). Bischoff et al. (2009) resurrected the name *M. brunneum*. Genomic studies showed that *M. brunneum* clustered with *M. brunneum sensu stricto* among other species to form a distinct clade (Pattemore et al. 2014). *M. brunneum* was shown to be pathogenic to *R. annulatus*, another cattle fever tick species of grave concern to U.S. animal agriculture (Miller et al. 2012), under field conditions (Samish et al. 2004).

The laboratory results shown here are in agreement with previous reports where non-commercial preparations of *M. brunneum* were tested. Camargo et al. (2012) reported 93% larval mortality at a dosage of 1 x 10^8 cfu/ml concentration *in vitro* against *B. microplus*. Our results were very similar and only slightly better than the 90% *in vitro* larval mortality reported by Ojeda-Chi et al. (2010) at the same concentration.

Tests with engorged female *B. microplus* resulted in 50% mortality at 6 d post-inoculation. However, mortality against engorged adult females tended to occur after much oviposition was achieved and with minimal impact on egg hatch. A similar effect was noted before. Ojeda-Chi et al. (2010) reported that with concentrations inducing 100% mortality of the adults, the numbers of eggs were reduced by only 39-55%. Similarly, Guedes-Frazzon et al. (2000) reported 53% reduction in oviposition but no effect on egg viability. *Metarhizium brunneum* is apparently more pathogenic to *B. annulatus* than to *B. microplus* because 85 -100% mortality in engorged female ticks was observed between 7 and 10 d post-treatment, and tick egg laying was prevented or reduced several days before death. (Gindin et al. 2001; Samish et al. 2004).

Scientific and technological advances are helping realize the potential to use *M. brunneum* formulations as part of integrated cattle fever tick management strategies. A formulation commercially available in Brazil aided in the control of *B. microplus* populations (Camargo et al. 2016). Angel-Sahagun et al. (2010) achieved encouraging results against *B. microplus* in field trials in tropical Mexico. Experimental formulations of *M. brunneum* tested under field conditions were shown to be effective in controlling *B. annulatus* (Samish et al. 2004). Environmental factors within the permanent quarantine zone of the Cattle Fever Tick Eradication Program (Wang et al. 2016) such as humidity may be important in considering the timing of applications. Further research would be required to define those conditions to use formulations of *M. brunneum* on cattle or in the environment for integrated cattle fever tick eradication (Pérez de León et al. 2012; Behle and Jackson 2014; Webster et al. 2015). The *M. brunneum* strain F52 formulation commercially available in the U.S. provides the opportunity to test its efficacy following pasture treatment taking into consideration the evaluation of factors such as effects on non-target arthropods, and palatability of treated forage to cattle.

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