Dose-response relationship of *Beauveria bassiana* (Balsamo) Vuillemin on *Rhipicephalus microplus* ticks

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Research

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Abstract

Background

Isolation and optimization are the primary step in the development of fungal pathogens for biological control agents. In this study, three indigenous strains of *Beauveria bassiana* (*Bb-27*, *Bb-40* and *Bb-41*) and one commercial strain (*Eco-Bb*) were tested for dose/response relationship of the fungus against *Rhipicephalus microplus* ticks.

Results

We used *R. microplus* larvae of 2 weeks of age, and exposed them to four conidial concentrations: 1×10^3 , 3×10^5 , 4×10^7 , 3.2×10^8 conidia.mL⁻¹ of each strain. Water was used as a control. Larval mortality was significantly affected by the type of formulation. There was a positive relationship between the concentration of the formulation and mortality of the treated ticks. All the ticks treated with the formulation containing 3.2×10^8 conidia.mL⁻¹ resulted in high levels of mortality.

Conclusions

From this study, it can be concluded that, because of the diversity in the performance of *B. bassiana* strains, optimization of the formulation for different Isolates of *B. bassiana* can be achieved through scrutinizing the virulence of the Isolates.

1 Background

Ticks such as the Southern cattle tick, *Rhipicephalus microplus* (Ixodida: Amblyommidae) are blood-feeding arthropods that parasitize livestock worldwide (De León et al. 2020). They are major livestock parasites and can transmit pathogens that cause diseases of economic importance in cattle (Springer et al. 2020; Ghafar et al. 2020). Control of ticks is, therefore, a major concern for all livestock farmers. To date, the control of ticks has been largely dependent upon the use of acaricides (Rodriguez-Vivas et al. 2018; Nwanade et al. 2020). However, due to the emergence of tick populations resistant to the acaricides (Brito et al. 2011), and concerns on food and environmental pollutions (Harrison and Wilmshurst 1973; George and Davey 2004; Shanmuganath et al. 2021), new approaches are being sought for effective tick control.

Recently entomopathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin has been used as an alternative to acaricides. Entomopathogenic fungi are natural enemies of arthropods. These entomopathogenic fungi invade the host by penetrating the outer surface of the host, hence, they not only infect the blood-sucking stage of the tick but also parasitize the non-feeding stages of the arthropod. Besides, the fungi also have a wide host range (De

Faria and Wraight 2007). The process of infection of the tick by the fungus starts with the formation of germ tubes that penetrate through the outer surface of the tick. However, factors such as the viability of the conidia and virulence factors that are specific to each isolate affect the pathogenicity of the isolate (Soetopo 2004). Therefore, isolating and selecting a largely efficacious strain is the first step in the development of a fungal biological control agent (BCA).

Once the most virulent strain has been isolated, utilising the optimal mode of inoculation is necessary for successful treatment. Dipping is one of the most commonly used modes of application to control ticks. However, this method has its flaws because: (i) it is expensive because it requires a large number of conidia for the suspension to completely dip the cow in the formulation; and (ii) it is not practical when the pasture also has to be treated against the non-parasitic stage of the tick. One of the efficient and practical application methods to treat for all life cycle stages of the ticks is spraying using ultra-low volume application equipment. Therefore, during laboratory tests, it is important to adopt a suitable method to apply the formulation that can be successfully replicated in the field. To do so, the formulation of the BCA (fungus, water, oil and adjuvant) must be optimised to develop a cost-effective and virulent formulation against the targeted host. Dose-response relationship assessment is one of the effective method used to optimise the formulation. In previous studies, we isolated three strains of *B. bassiana* with a high degree of virulence against *R. microplus*. The aim of this study was to conduct several concentration-mortality tests to determine the optimal concentration of conidia of formulated *B. bassiana* needed to control *R. microplus*.

2 Methods

2.1 Ethics statement

All experiments using live animals were performed in accordance with the University of KwaZulu-Natal guidelines on animal experimentation and were approved by the Committee of Ethics of Animal (REF: AREC/018/017D). Laboratory bioassays were carried out at Biocontrol Research Laboratory, College of Agriculture, Engineering and Science, School of Agricultural, Earth and Environmental Sciences, Pietermaritzburg campus, South Africa.

2.2 Ticks

Engorged *R. microplus* ticks were collected from cattle in Ukulinga Research Farm in 2018. Engorged females were placed into Petri dishes and incubated under laboratory conditions, at 27 ± 1 °C and RH of $\geq 80\%$ to allow oviposition. Eggs were then transferred into plastic cups (30 mm radius, 20mm height) with perforated lids. Live larvae were used for the concentration-mortality virulence assessment.

2.3 Fungal isolates

Four strains of *B. bassiana* were used in the present study. Three strains were locally isolated in our laboratory, whereas the fourth one - Eco-Bb® - was a commercial strain obtained from Plant Health Products (Pty) Ltd. Fungal isolates were cultured in Potato Dextrose Agar (PDA) medium to provide

sufficient conidia for further research. To prepare the PDA medium, 39 g of commercial Potato Dextrose Agar powder (Merck, Darmstadt, Germany) amended with 1 mL of Dodine (Merck, Darmstadt, Germany) and 0.05 g of Chloramphenicol (Merck, Darmstadt, Germany) was added into 1000 mL of distilled water. Then the mixture was boiled while mixing to dissolve completely. To sterilise the media the mixture was autoclaved at 121°C for 15 minutes. Finally, the solution was aseptically dispensed into sterile Petri dishes. The petri dishes (90-mm) were incubated at $25 \pm 2^\circ\text{C}$ in complete darkness. Conidia were harvested from 3-week-old cultures with a sterile spatula.

2.4 Concentration-mortality virulence bioassays of selected isolates

Fungal virulence towards ticks was determined using formulations of varying conidial concentrations. We used *R. microplus* larvae of 2 weeks of age, and exposed them to four conidial concentrations: 1×10^3 , 3×10^5 , 4×10^7 , 3.2×10^8 conidia.mL⁻¹ of each strain; distilled water containing 0.05% breackthrou® and 10% avocado oil was used as a control. All the formulations were prepared in sterile distilled water containing 0.05% breackthrou® and 10% avocado oil. Conidial counts were adjusted to the desired concentration after counting the conidia using a haemocytometer (Neubauer-improved: Hirschmann®).

Fifteen larvae of *R. microplus* were placed in plastic cups with perforated lids. Each cup was sprayed using a hand-held, finger-tip sprayer from a 60-mL, travel-size bottle (Sprayco, Livonia, MI, USA) affixed to a 15-mL conical Fisherbrand centrifuge tube. Trials at each concentration were replicated three times. All treatments were then incubated at $27 \pm 1^\circ\text{C}$ and $\text{RH} \geq 80\%$. All larvae were individually examined under a dissecting microscope for the detection of fungal infection. Larvae were considered to be dead from the absence of movement after stimulation, and observation of mycelium emerging from the cuticle. Post-treatment mortality was recorded daily. Dead larvae from all treatment groups were incubated at $25 \pm 1^\circ\text{C}$ and $\text{RH} \geq 80\%$ for 20 days to allow fungal growth.

2.5 Statistical Analysis

The mortality caused by each treatment was recorded daily for 12 days. Corrected mortality was calculated using the formula developed by Abbott recommended by the FAO (Abbott, 1925; FAO, 2004). Then the data were subjected to one way ANOVA. Values of $p < 0.05$ were considered significant. Where there is an overall statistically significant difference among groups, post hoc analysis (Tukey) was performed to indicate the difference between groups. The LC_{50} and LC_{90} were determined using probit analysis and the data were subjected to two-way ANOVA. Means were considered to be statistically different at $P < 0.05$.

3 Results

3.1 Virulence of four strains of *Beauveria bassiana* against *Rhipicephalus microplus* larvae

The virulence of 4 strains of *B. bassiana* applied at varying concentration and control groups to larvae of *R. microplus* are presented in Table 1. All isolates of *B. bassiana* were pathogenic to *R. microplus* at all concentrations but with varying degree of virulence. Generally, the pathogenicity of the fungus for all strains increased as the concentration of the conidia increased (Fig. 1 to 4). Those ticks treated with Eco-*Bb* at the concentration of 10^3 conidia.mL⁻¹ recorded the lowest mortality. Overall, the mortality of the treated ticks increased 12 days post-treatment. However, treatment with *Bb*-40 at the concentration of 10^8 conidia.mL⁻¹ was the best performing formulation 12 days after treatment. Mortality in the control group was less than 10%. Larval mortality was significantly affected by the formulation at 12 days post-treatment. Twenty days after incubation many of the infected larvae of *R. microplus* were covered with fungal hyphae.

Table 1
Virulence of four strains of *Beauveria bassiana* against *Rhipicephalus microplus* larvae

Treatment	% Mortality (Mean)
Control	3.5 ^a
1x10 ³ Eco- <i>Bb</i>	12.33 ^a
1x10 ³ <i>Bb</i> -27	17.67 ^{ab}
1x10 ³ <i>Bb</i> -40	25.67 ^{ab}
3x10 ⁵ Eco- <i>Bb</i>	26.67 ^{abc}
3x10 ⁵ <i>Bb</i> -27	39.83 ^{bcd}
1x10 ³ <i>Bb</i> -41	40.0 ^{bcd}
4x10 ⁷ Eco- <i>Bb</i>	41.17 ^{bcd}
3x10 ⁵ <i>Bb</i> -41	42.17 ^{bcd}
3x10 ⁵ <i>Bb</i> -40	52.17 ^{cde}
4x10 ⁷ <i>Bb</i> -27	52.17 ^{cde}
3.2x10 ⁸ <i>Bb</i> -27	61.0 ^{def}
3.2x10 ⁸ Eco- <i>Bb</i>	63.17 ^{def}
4x10 ⁷ <i>Bb</i> -41	64.5 ^{def}
4x10 ⁷ <i>Bb</i> -40	74.5 ^{ef}
3.2x10 ⁸ <i>Bb</i> -41	78.67 ^f
3.2x10 ⁸ <i>Bb</i> -40	92.17 ^g
P Value	0.001
F Value	10.59
SE	2.87
DF	16

3.2 Dose-response assessment for four strains of *Beauveria bassiana* in virulence bioassays against unfed larvae of *Rhipicephalus microplus*

The lethal concentrations, LC₅₀ and LC₉₀ are shown in Table 2. The lethal concentration (LC₅₀) was not significantly affected by isolate at a 95% confidence level. Generally all isolates needed the low concentration to kill 50% of the larvae. The results of LC₉₀ shows that substantially higher concentration of the conidia is required to achieve 90% mortality of *R. microplus* larvae in the short term. However, it is not clear why there was no significant difference among groups.

Table 2

The lethal concentrations of *Beauveria bassiana* conidia required to cause 50 and 90% mortality (LC₅₀ and LC₉₀) of unfed larvae of *R. microplus* 12 days after treatment

LC	Isolate	Mean	SD	95% Confidence Interval for Mean		P-value
				Lower Bound	Upper Bound	
LC ₅₀	<i>Bb-27</i>	1.86 x 10 ⁷	1.58 x 10 ⁷	1.99 x 10 ⁶	3.52 x 10 ⁷	0.059
	<i>Bb-40</i>	4.82 x 10 ⁶	1.17 x 10 ⁷	-7.42 x 10 ⁶	1.71 x 10 ⁷	
	<i>Bb-41</i>	1.16 x 10 ⁶	1.16 x 10 ⁶	-5.91 x 10 ⁴	2.37 x 10 ⁶	
	<i>Eco-Bb</i>	1.13 x 10 ⁸	1.51 x 10 ⁸	-4.51 x 10 ⁷	2.71 x 10 ⁸	
LC ₉₀	<i>Bb-27</i>	1.48 x 10 ¹⁷	3.58 x 10 ¹⁷	-2.28 x 10 ¹⁷	5.23 x 10 ¹⁷	0.404
	<i>Bb-40</i>	3.24 x 10 ⁹	4.80 x 10 ⁹	-1.81 x 10 ⁹	8.28 x 10 ⁹	
	<i>Bb-41</i>	2.67 x 10 ¹³	3.81 x 10 ¹³	-1.33 x 10 ¹³	6.67 x 10 ¹³	
	<i>Eco-Bb</i>	7.12 x 10 ¹³	7.19 x 10 ¹³	-4.24 x 10 ¹²	1.47 x 10 ¹⁴	

4 Discussion

Many laboratory studies have been conducted on the use of entomopathogenic fungi for the biological control for livestock ticks (Frazzon et al. 2000; Sun et al. 2011; Camargo et al. 2012; Ma et al. 2013). In the current study, we observed that the fungus is highly virulent on unfed larvae of *R. microplus* but with varying degrees of pathogenicity among the different isolates tested. All isolates caused more than 60% mortality of unfed larvae of *R. microplus*, 12 days after treatment with 10⁸ conidia.mL⁻¹. However, no isolate caused mortality greater than 50%, six days after application with the same concentration. This means that all the tested isolates need more time to infect the larvae. Furthermore, the mortality of the larvae increased as the concentration of the conidia in the formulation increased, demonstrating dose-dependent mortality in *R. microplus* larvae. The question then is whether a higher conidial concentration was required to overcome the tick defence system during germination and proliferation stage, or just a large number of conidia are required to increase the chance of conidia to stick on the cuticle of the tick. The same trend in the dose/response relationship was observed by Frazzon et al. (2000) who found that all *B. bassiana* isolates tested were pathogenic to ticks, but the isolates had varying degrees of virulence

against *R. microplus* larvae. Samish et al. (2001) also observed variation in the pathogenicity of various entomopathogenic fungi to different developmental stages of *R. microplus*. Under laboratory conditions, the variation in the virulence of the fungus within the species could be explained by the ability of the strain to penetrate the cuticle of the tick. Enzymes such as proteases (Joshi et al. 1995) and chitinase (Fang et al., 2005) are important virulence factors for *B. bassiana*.

As compared to the mortality observed in our previous study (unpublished), the mortality induced by isolate *Bb-27* was low (61 vs 91) for same concentration (10^8 conidia.mL⁻¹) 12 days post-treatment. This discrepancy could have resulted from either culturing conditions or attenuation of virulence due to successive subculturing on artificial media (Butt and Goettel 2000)

The process of infection started after about four days. But four days post-treatment the mobility of the infected larvae was significantly affected while the non-infected larvae were moving vigorously. Unlike our observation during the isolation of entomopathogenic fungi using *Galleria mellonella*, the cadavers did not show any change in colour in contrast to the non-infected larvae. But five days after further incubation, some of the cadavers start to be covered by white hyphae. However, even after incubation for 20 days, not all larvae were covered by the hyphae. In contrast, Samish et al. (2001) reported that two days post-treatment the colour of the larvae darkened, followed by immobilisation of the treated ticks indicating an infection in larvae. After incubation for 72 hours, sporulation started to appear on the surface of the larvae. In another study, Abdigoudarzi et al. (2009) reported that sporulation from the tick surface was not observed. In our studies case, since some of the cadavers showed sporulation in their surface, Secondary epizootics or horizontal infection can increase the overall infection level of a tick population by the entomopathogenic fungi. Klinger et al. (2006), determined the likelihood of secondary infection of adult Colorado potato beetles (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) in the presence of *Beauveria bassiana* sporulating on cadavers. Their results indicated that significant likelihood of horizontal infection of adults (30–70% of horizontal infection) from the cadavers with the presence of sporulation.

5 Conclusions

Given the diversity in the performance of *B. bassiana* strains, a selection of virulent isolates is crucial for the development of efficacious bio-acaricides. The present study also demonstrated a positive dose/response relationship. Further studies are required to validate whether there is indeed a horizontal infection between cadavers and new uninfected tick populations. Additionally, field trials are required to confirm these results under field conditions.

6 List Of Abbreviations

<i>B. bassiana</i>	<i>Beauveria bassiana</i>
L	Liter

mL	Milliliter
RH	Relative Humidity
g	Gram
mg	Milligram
<i>R. microplus</i>	<i>Rhipicephalus microplus</i>

Declarations

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal

Ethical approval and consent to participate: All experiments using live animals were performed following the University of KwaZulu-Natal guidelines on animal experimentation and were approved by the Committee of Ethics of Animal (REF: AREC/018/017D).

Consent for Publication: All authors have approved the manuscript and agree with its submission to Egyptian Journal of Biological Pest Control.

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Authors Contribution: Prof Laing Conceived the research and reviewed the manuscript. Dr. Zeina designed the analysis; Collected the data; Performed the analysis and wrote the paper.

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Figures

Figure 1

Mortality of *R. microplus* larvae treated with formulations of four strains of *Beauveria bassiana*: Bb-27 (From Day 1 to day 12)

Figure 2

Mortality of *R. microplus* larvae treated with formulations of four strains of *Beauveria bassiana*: Bb-40 (From Day 1 to day 12)

Figure 3

Mortality of *R. microplus* larvae treated with formulations of four strains of *Beauveria bassiana*: Bb-41 (From Day 1 to day 12)

Figure 4

Mortality of *R. microplus* larvae treated with formulations of four strains of *Beauveria bassiana*: Eco-Bb (From Day 1 to day 12)

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