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Optimizing modes of inoculation of *Rhipicephalus* ticks (Acari: Ixodidae) with a mitosporic entomopathogenic fungus in the laboratory

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Abstract The process of strain selection is an important step in the development of insect pathogens for biological control. Bioassays were conducted in the laboratory to evaluate the efficacy of different methods of inoculation using *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae) as a model. Initially, an oil-based formulation of *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales) titred at 10^9 conidia ml^{-1} was applied to *R. pulchellus* adults using a Burgerjon spray tower or a microapplicator. Inoculation by microapplicator yielded poor results (25.0% tick mortality) compared to Burgerjon's spray tower (52.3% tick mortality), although the mean number of fungal conidia on *R. pulchellus* adults was lower ($1.5 \times 10^4 \pm 1.1 \times 10^3$ conidia ml^{-1}) after spraying by Burgerjon's spray tower compared to 1×10^6 conidia ml^{-1} obtained with the microapplicator. Thus, inoculation by Burgerjon's spray tower was selected for further investigations. Different modes of inoculation were tested and included direct spray of inoculum on the tick and substrate (SS), direct spray on the substrate and tick followed by transfer of the tick to clean uncontaminated Petri dish (SP) or indirect inoculation of ticks through substrate (SW). The LC_{50} values following contamination of nymphs ($LC_{50} = 1.4 \times 10^7$ conidia ml^{-1}) and adults ($LC_{50} = 6.7 \times 10^7$ conidia ml^{-1}) in SS were significantly lower compared to SP; nymphs ($LC_{50} = 5.7 \times 10^8$ conidia ml^{-1}) and adults ($LC_{50} = 5.3 \times 10^9$ conidia ml^{-1}) and SW; nymphs ($LC_{50} = 5 \times 10^8$ conidia ml^{-1}). Although the LC_{50} value in SS was the lowest, it recorded the highest tick mortality among control ticks (24.2% at 2 weeks post-treatment) and (23.3% at 3 weeks post-treatment) in nymphs and adults respectively compared to SP (2.5 and 5.8%, respectively) and SW (0.0 and 0.0). Results show that among the modes of inoculation tested, SP was the most appropriate for inoculating *R. pulchellus* adults. SW and SP were identified as appropriate techniques for infecting the *R. pulchellus* nymphs with conidia formulated in oil.

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Abbreviations

SS Direct spray of inoculum on the tick and substrate

SP Direct spray on the substrate and tick followed by transfer of the tick to clean uncontaminated Petri dish

SW Indirect inoculation of ticks through substrate

Introduction

Strain selection is the primary step in the development of fungal pathogens for biological control (Soper and Ward 1981). Several modes of inoculation have been used to evaluate the virulence of mitosporic Hyphomycetes. These include spraying conidia directly onto the target; exposing arthropods to treated leaves; dipping arthropods into titrated conidial suspensions; and treating the substrate (Hall and Papierok 1982). Another technique was developed for screening fungal pathogens against locust and it consists of applying inoculum on the pronotum of the insect (Prior et al. 1995). The most common method used for inoculation of ticks has been by immersing them into titrated conidial suspensions (Frazzon et al. 2000; Onofre et al. 2001; Samish et al. 2001; Kirkland et al. 2004). Dipping assays have shown that *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales) is a virulent pathogen of members of the genus *Rhipicephalus* (Acari: Ixodidae) (Samish et al. 2001; Gindin et al. 2002; Maniania et al. 2007). However, inoculation of ticks by dipping optimizes exposure to a pathogen and may not allow the most virulent strains to be readily differentiated from the less virulent ones. Dipping in conidial suspension can cause blockage of the spiracles of the host, resulting in high mortalities (Soarés 1982). Furthermore, dipping of the ticks in conidial suspension does not closely "mimic" the already established ULV (ultra low volume) spraying technique, which might be less expensive and practical for application of fungal suspensions over large area of grass vegetation infested with ticks that quests from the vegetation ("ambusher" ticks). In reality, these ticks will acquire the conidia directly or indirectly from the foliage when crawling over the contaminated substrate in a ULV spray application with reduced chances of excessive fungal contamination as is the case with immersion.

The development of oil-based formulations for entomopathogenic fungi has improved the control of many arthropod pests (Lomer et al. 2001; Kaaya and Hassan 2000; Shi et al. 2008). Pure oils are compactible with the established ULV spray technique that is suitable for large-scale field application (Polar et al. 2005). Considering that large areas may require treatments, as well as the costs involved with spore production and labour, pure oil formulation may be more effective compared to 10% water–oil emulsion; since it is compatible with ULV application technique. Most workers investigating the biological control of ticks using *Metarhizium* have only reported using aqueous formulations (Mwangi et al. 1995; Monteiro et al. 1998a, b; Correia et al. 1998). Others have evaluated pathogenicity of entomopathogenic fungal isolates suspended in oil–water emulsions (Kaaya and Hassan 2000; Leemon and Jonsson 2008). According to previous works, only one other study has investigated pathogenicity of *M. anisopliae* suspended in pure oils against ticks (Polar et al. 2005). Optimizing inoculation procedure for infecting ticks with

conidia formulated in pure oil may facilitate strain selection in the context of ULV application of mycoacaricide for tick control. Here, different methods of applying *M. anisopliae* onto nymphs and adults of the hard tick *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae) were evaluated in laboratory in order to define superior methods to screen for the most effective fungal isolates.

Materials and methods

Tick

Ticks were obtained from the Animal and Quarantine Rearing Unit at *icipe*. The initial colony was established from adult ticks collected from the vegetation in Mwea Game Park reserve, Kenya, in 2006. Larvae, nymphs, and females were fed on New Zealand white rabbits and incubated in clear Perspex chambers at 26°C ± 1 and 85% ± 5 RH for 12:12 L:D photoperiod. Three to four week-old unfed adult ticks and 2–3 week old unfed nymphs were used for this study.

Fungus

Metarhizium anisopliae (ICIPE 60) used in this study was obtained from the *icipe's* Arthropod Germplasm Centre. The strain was originally isolated from soil in Kakelo Kisumu, Kenya in 1996 and was previously found to be infective against *R. pulchellus* (Personal communication). The fungus was cultured on SDA plates at 26 ± 2°C. The virulence of the fungal strain was maintained by a single passage through the *R. pulchellus* (Schaerffenberg 1964). Two to three week-old aerial conidia of *M. anisopliae* were harvested by scraping and suspended into corn oil (CHEF cooking oil, Premier Oil Mills) in a universal bottle containing glass beads. The suspension was then mixed vigorously in a vortex shaker for more than 5 min to homogenize the suspension. Conidial concentration was determined using an improved Neubauer haemocytometer and different test concentrations (10^6, 10^7, 10^8, 10^9 and 10^{10} conidia ml^{-1}) were obtained by serial dilutions in corn oil. Viability of the conidia was determined before each bioassay by spread-plating 0.1 ml of conidial suspension titrated at 1×10^6 conidia ml^{-1} onto SDA plates which were examined under a light microscope 18 h later. Conidial germination was determined from 100-spore counts with four replicates. Germination rates of >90% were regularly obtained.

Attempts to include petroleum oils (kerosene and diesel) as carriers or in a mixture with corn oil in the current study was abandoned because thick swirling cloud of droplets was produced following spray by Burgerjon's spray tower (Burgerjon 1956), which may contaminate the laboratory.

Inoculation procedures

The experiments were divided into two parts; part 1 and part 2. In part 1, two inoculation methods were tested: (i) via a Burgerjon spray tower; (ii) using a microapplicator. Oil-based formulation of *M. anisopliae* (ICIPE 60) titred to 10^9 conidia ml^{-1} was applied to *R. pulchellus* adults using both application methods. Since comparatively lower infection rates were obtained following treatment by microapplicator in Part 1, subsequently in Part 2 experiment, all further treatments were delivered via Burgerjon's sprayer. In order to

estimate the number of propagules on each tick 1 unfed male tick (size of tick was approximately 4 mm long including mouth parts) and 1 nymph were immediately placed into separate 10-ml vial containing 0.05% Triton X-100 after each inoculation procedure was completed. Vials were subjected to shaking by a vortex shaker for 5 min to dislodge conidia from the tick surface. The number of conidia ml^{-1} was determined using an improved Neubauer haemocytometer. The treatment consisted of 25 replicates per inoculation procedure. It is worth noting this method was not sensitive enough to detect the number of propagules on ticks at lower concentrations of conidial suspensions, especially for the nymphal stage.

Microapplication

Adult individual ticks were inoculated with 1 µl of conidial suspension titred at 10^9 conidia ml^{-1} formulated in oil applied around the anterior region on the joint between the idiosoma and basis capitulum using a 1 ml-syringe fixed to a microapplicator (Arnold Hand Microapplicator Burkard Manufacturing, Rickmansworth England). In the control, ticks were treated with oil without conidia. Test-ticks were transferred to 9 cm-diameter Petri dish after treatment and maintained at 25 ± 1°C and 85 ± 5% RH. Mortality was recorded at 4 weeks post-treatment. Mortality caused by fungus was confirmed by microscopic examination of hyphae and spores on the body of dead ticks following incubation under high humid condition for 4–5 days. There were 20 ticks per replicate and 6 replicates in total per treatment group.

Burgerjon's spray tower

The tower was fitted with an air-atomising nozzle connected to a regulator valve providing a constant airflow under 4 bar pressure, resulting to a deposit of approximately 3.8×10^6 conidia cm^2. In the initial bioassays in Part 1, 10 ml of conidial suspension titred at 1×10^9 conidia ml^{-1} was sprayed directly on ticks placed on a Petri dish (9 cm in diameter) lined with filter paper and ticks immediately transferred to clean Petri dishes (9 cm in diameter). The ticks were immobilised by placing the Petri dishes in which they were held on crushed ice and were placed with the dorsal side uppermost while spraying. In the subsequent bioassays, three methods were used to infect ticks with the inoculum. The inoculation methods were designed to "mimic" the possible routes of inoculation (i.e. directly, indirectly or a combination of both direct and indirect routes) of ticks with fungus in an oil suspension following ULV spray-application under field conditions. The criteria for selecting the most appropriate method for assaying fungal isolates suspended in pure oil was based on mortality in both the control and fungus treated ticks.

i. Direct spray of ticks and the substrate (filter paper lined at the bottom of a Petri dish) on which ticks were placed. Ticks were maintained in the treated Petri dish for 12 h before being transfer to clean Petri dishes (SS).
ii. Direct spray of ticks and substrate as above but ticks were transferred to clean dishes immediately (between 0 and 5 min) after contamination was completed (SP).
iii. Contamination of the substrate (filter paper). Petri dish lined at the bottom with a filter paper was sprayed and were then placed in an upside down position to allow ticks to climb up to the contaminated substrate naturally. Ticks were then introduced into contaminated dishes where they were held for 12 h before removal to clean dishes (SW).

In all cases ticks were maintained at 25 ± 1°C and 85 ± 5% RH for 2 weeks (nymphs) and 3 weeks (adults). In the control treatments, ticks were treated with oil only. Treatments were block randomized and consisted of 20 ticks per replicate and the experiment was replicated 6 times.

Data analyses

Tick mortality was adjusted (Abbot 1925). ANOVA was performed on arcsin-transformed Abbott percentage mortality data, log base 10 (×) transformations of number of spores on tick and means separated by Tukey's (HSD) test. Comparison for significance was at $P = 0.05$ significance level. LC_{50} was determined using probit analysis and their 95% confidence intervals (IC) were used to evaluate significant difference of LC_{50} between different modes of inoculation. The analyses were performed using the SAS package (SAS 2001).

Results

Part 1 experiment; comparing different application methods

Control mortality in Part 1 experiment was 4.2 and 7.5% when the control treatment was applied by microapplicator and Burgerjon's spray tower, respectively. Mortality from the fungal treatments was 25.0 and 52.3% following application by microapplicator and spray tower, respectively, at 4 weeks post-treatment (Fig. 1). The estimated mean number of fungal propagules on *R. pulchellus* adults was $1.5 \times 10^4 \pm 1.1 \times 10^3$ conidia ml^{-1} after spraying by Burgerjon's spray tower compared to 1×10^6 conidia ml^{-1} obtained with the microapplicator.

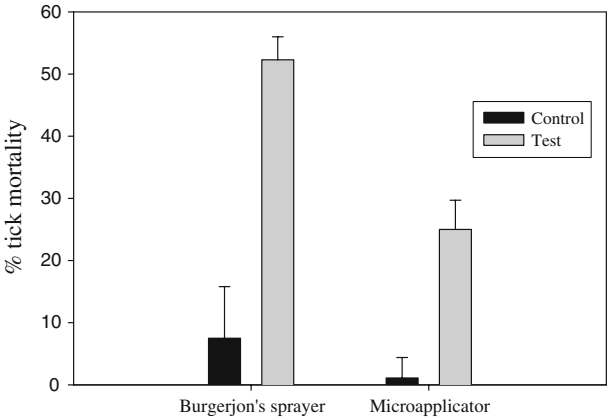

Fig. 1 Mortality (%) of adult *R. pulchellus* following inoculation with oil suspension of *M. anisopliae* titred at 1×10^9 conidia ml^{-1} applied by Burgerjon's spray tower (direct spray on substrate and tick, tick then transferred to a clean Petri dish), or using a microapplicator at 4 weeks post-treatment

Table 1 Percentage mortality (mean ± SE) of *R. pulchellus* nymphs to varying concentrations of pure oil formulation of *M. anisopliae* following different methods of exposure to conidia, 14 days post-treatment

Concentration of conidia ml^{-1} before spraying on ticks	Mode of exposure		
	SS	SW	SP
Control	24.2 ± 1.5	0.0 ± 0.0	2.5 ± 1.1
10^6	29.3 ± 10.4 bA	0.0 ± 0 cB	25.7 ± 5.8 Ba
10^7	28.3 ± 8.9 bA	2.5 ± 2.5 cA	26.8 ± 15 Ba
10^8	100.0 ± 0 aA	14.2 ± 2.4 bC	57.6 ± 16.1 abB
10^9	100.0 ± 0 aA	96.7 ± 2.1 aA	66.1 ± 14.7 abB
10^{10}	100.0 ± 0 aA	100.0 ± 0 aA	100.0 ± 0 Aa
LC_{50} (CI)	1.4×10^7 conidia ml^{-1} (1.2×10^7–1.6×10^7 conidia ml^{-1})	5×10^8 conidia ml^{-1} (4.7×10^8–5.3×10^8 conidia ml^{-1})	5.7×10^8 conidia ml^{-1} (5.2×10^8–6.3×10^8 conidia ml^{-1})

Means with same lowercase letter in each column and uppercase letter in each row are not significantly different at $P = 0.05$ following analysis with Tukey's test. CI signifies confidence interval

SS direct spray of the inoculum on the nymphs and substrate, *SP* direct spray on substrate and nymphs, nymphs then transferred to a clean Petri dish, and *SW* exposure of nymphs to contaminated substrate

Nymph assays

Mortality of nymphs that were directly exposed to a fungal spray (SS) was 24.2% in the control but ranged from 29.3% (lowest concentration) to 100% (highest concentration), 2 weeks after fungus treatment (Table 1). Mortality of nymphs that were sprayed and transferred into clean Petri dishes (SP) was 2.5% in the control and ranged from 25.7% (lowest concentration) to 100% (highest concentration) in the fungus-treatment, 2 weeks post-treatment (Table 1). Mortality of nymphs that were exposed indirectly (SW) was 0.0% in the control and ranged from 0.0% (lowest concentration) and 100% (highest concentration) in the fungus treatment, 2 weeks post-treatment (Table 1). Mortality of *R. pulchellus* nymphs increased significantly ($df = 4,\ 25;\ P < 0.001$) with increasing concentrations of conidia irrespective of the method of exposure (SS, SW and SP). The fungus was most effective when application was by direct exposure (SS) ($LC_{50} = 1.4 \times 10^7$ conidia ml^{-1} and CI = 1.2×10^7–1.6×10^7 conidia ml^{-1}) followed by SP ($LC_{50} = 5.7 \times 10^8$ conidia ml^{-1} and CI = 5.2×10^8–6.3×10^8 conidia ml^{-1}) and SW ($LC_{50} = 5 \times 10^8$ conidia ml^{-1} and CI = 4.7×10^8–5.3×10^8 conidia ml^{-1}), 14 days post-treatment. However, there was a significantly higher ($df = 2, 74$; $P < 0.0001$) mean number of fungal propagules on ticks after spraying 10^{10} conidia ml^{-1} by SS ($1.5 \times 10^4 \pm 0.9 \times 10^3$ conidia ml^{-1}) compared to SP ($9.9 \times 10^3 \pm 6 \times 10^2$ conidia ml^{-1}) and SW ($4.4 \times 10^3 \pm 3.9 \times 10^2$ conidia ml^{-1}) (Table 2).

Adult assays

Mortality of adults that were directly exposed to fungal sprays (SS) was 23.3% in the control and ranged from 25.4% (lowest concentration) to 100% (highest concentration) in the fungus treatments, 3 weeks after spraying (Table 3). Mortality of adults that were directly exposed to fungal spray and then transferred into clean plates (SP) was 5.8% in the control and ranged from 17.0% (lowest concentration) to 70.9% (highest concentration) in

Table 2 Number of *M. anisopliae* spores (mean ± SE) on nymphs and adults of *R. pulchellus* after application of oil suspension of 10^{10} conidia ml^{-1} by different modes of exposure to conidia

Life stage of tick	Number of spores on each tick (mean ± SE) Mode of exposure		
	SS	SW	SP
Nymph	$1.5 \times 10^4 \pm 0.9 \times 10^3$ A	$4.4 \times 10^3 \pm 3.9 \times 10^2$ C	$9.9 \times 10^3 \pm 6 \times 10^2$ B
Adult	$1.3 \times 10^5 \pm 1.1 \times 10^4$ A	$2.3 \times 10^4 \pm 1.3 \times 10^3$ C	$9.2 \times 10^4 \pm 4.5 \times 10^3$ B

Mean with same uppercase letter in each row is not significantly different at 0.05 level of significance following analysis with Tukey's test

SS direct spray of the inoculum on the tick and substrate, *SP* direct spray on substrate and tick, tick then transferred to a clean Petri dish, and *SW* exposure of ticks to contaminated substrate

Table 3 Percentage mortality (mean ± SE) of *R. pulchellus* adults to varying concentrations of oil-based formulation of *M. anisopliae* following different methods of exposure to conidia, 21 days post-treatment

Concentration of conidia ml^{-1} before spraying on ticks	Mode of exposure		
	SS	SW	SP
Control	23.3 ± 2.7	0.0 ± 0.0	12.5 ± 2.5
10^7	25.4 ± 4.9 cA	0.0 ± 0 bB	17.0 ± 5.9 cA
10^8	72.0 ± 7.4 bA	0.0 ± 0 bC	31.8 ± 7.9 bcB
10^9	100.0 ± 0 aA	0.0 ± 0 bC	53.0 ± 11.7 abB
10^{10}	100.0 ± 0 aA	15.0 ± 3.2 aC	70.9 ± 5.8 aB
LC_{50} (CI)	6.7×10^7 conidia ml^{-1} (6.3×10^7–7.2×10^7 conidia ml^{-1})	$>1 \times 10^{10}$ conidia ml^{-1}	5.3×10^9 conidia ml^{-1} (4.9×10^9–5.8×10^9 conidia ml^{-1})

Means with same lowercase letter in each column and uppercase letter in each row are not significantly different at 0.05 level of significance following analysis with Tukey's test. CI signifies confidence interval

SS direct spray of the inoculum on the adult and substrate, *SP* direct spray on substrate and adult, adult then transferred to a clean Petri dish, and *SW* exposure of ticks to contaminated substrate

the fungus-treated lots, 3 weeks post-treatment (Table 3). Mortality of adults that were exposed to treated substrate (SW) was 0.0% in the control and ranged from 0.0% (lowest concentration) to 15.0% (highest concentration) in the fungus-treated lots, 3 weeks after treatment (Table 3). Mortality increased significantly ($df = 3, 20$; $P < 0.001$) with increasing concentrations in all the three techniques (SS, SW and SP) 3 weeks post-treatment (Tables 3). The fungus was most effective when application was by direct exposure (SS) ($LC_{50} = 6.7 \times 10^7$ conidia ml^{-1} and CI = 6.3×10^7–7.2×10^7 conidia ml^{-1}) followed by SP ($LC_{50} = 5.3 \times 10^9$ conidia ml^{-1} and CI = 4.9×10^9–5.8×10^9 conidia ml^{-1}) and ($LC_{50} > 10^{10}$ conidia ml^{-1}), 21 days post-treatment. The mean number of fungal propagules on ticks following direct spray (SS) ($1.3 \times 10^5 \pm 1.1 \times 10^4$ conidia ml^{-1}) of 10 ml of fungal suspensions titred at 10^{10} conidia ml^{-1} was significantly higher ($df = 2, 72$; $P < 0.0001$) compared to SP ($9.2 \times 10^4 \pm 4.5 \times 10^3$ conidia ml^{-1}) and SW ($2.3 \times 10^4 \pm 1.3 \times 10^3$ conidia ml^{-1}) (Table 2).

Discussion

Scant information on the optimization of assay techniques for *Rhipicephalus* ticks ("ambusher") and the use of pure oils as formulation agents for assaying entomogenous fungi against ticks was found in literature, prompting the current series of experiments. In the Part 1 experiment, application of fungi via a Burgerjon's spray tower resulted in higher mortality than inoculation with a microapplicator. The former technique was therefore selected for further studies. High mortality levels were recorded in the control treatments when nymph and adult ticks were exposed to sprays and maintained in the same Petri dish (SS). This may be explained by the toxic effect of oil to arthropods (Goettel and Inglis 1997; Moslim et al. 2004) and the prolonged exposure time compared to the other treatments. Previously, pure coconut oil caused high mortality in control-treated *R. microplus* (Polar et al. 2005). They suggested that high mortality caused by the pure coconut oil might have contributed to tick mortality induced by *M. anisopliae* formulated in coconut oil. Although mortality in the control-treated ticks can be accounted statistically, bioassay results may become meaningless if control mortalities are too high (Butt and Goettel 2000).

Significant differences in tick mortality following treatment with fungi were observed among the different methods of inoculation used, implying that the way the pathogen is applied can influence host mortality. For example, tick mortality recorded following exposure to spray via Burgerjon's spray tower was higher than in microapplicator, although the actual number of fungal propagules on tick was higher in the latter. Differences in techniques of contamination (immersing adults in a conidial suspension or placing the preimaginal stages on paper soaked with conidial) and life stages of ticks may induce different levels of infection (Samish et al. 2001). Distribution of conidia on the host is likely to differ according to the method of exposure, and successful infection is more likely via some parts of the cuticle than others (Fernandez et al. 2001). For example, penetrations of the cuticle at the many leg joints, mouthparts, spiracles or setae is easier than other parts of the tick cuticle (Arruda et al. 2005; Leemon and Jonsson 2008) and were more likely to be contaminated by droplets applied by the spray tower than the microapplicator. These parts could present easy points for germinating tubes and their exposure to spray droplets by Burgerjon's spray tower might have resulted in the higher tick mortality than the microapplicator where the conidial suspension was applied onto one joint, i.e. between the idiosoma and basis capitulum. In ticks, it has been observed that the setae and cuticular ridge are easily penetrable by the fungus (Leemon and Jonsson 2008). Scanning electron micrograph analysis of infected ticks showed that *M. anisopliae* conidia preferred attachment sites at certain junctions, like those present in the tick legs (Arruda et al. 2005). The cuticle is thinner in these areas (Sonenshine 1991). Cuticular folds in arthropods exoskeletons may also be sites of high moisture (Inglis et al. 2001) and previous observations suggested that conidial germination varied by body region (Kirkland et al. 2004).

The calculated LC_{50} values (obtained from the conidial suspensions before application on ticks) showed that higher infection levels were obtained when nymphs and adults of *R. pulchellus* were sprayed directly and maintained on treated substrate for 12 h (SS) compared to when sprayed directly and transferred to clean dishes (SP) or indirectly treated by exposure to contaminated substrate (SW). The higher dose of the acquired inocula per tick in SS could be responsible for the higher mortality recorded since mortality was dose-dependent. Tick mortality increases with conidial concentrations (Zhioua et al. 1997; Frazzon et al. 2000). Increase in conidia concentrations could have led to corresponding increases in the actual number of conidia deposited on the tick, thus inducing higher

infection levels. Even though nymphs acquired lower dose of fungal propagules than the adults, they were relatively more susceptible compared to the adults in the three inoculation methods. The relatively smaller size of nymph and the lack of thick cuticle compared to adult tick could have favoured the high levels of infection. From the results obtained in the current study, it appears that the number of spores on the cuticle must reach a certain threshold to induce high levels of tick mortality. Sharp increase in mortality of *R. microplus* engorged larvae and females at concentrations above 10^6 conidia ml^{-1} and minimal mortality below the concentration was recorded in the past (Zhioua et al. 1997). It is thus important researchers obtain estimates of the number of propagules on tick during bioassay to minimize variation in results obtained in different laboratories with the same fungal isolates, since the method of application influences the actual number of conidia deposited on ticks.

Based on the behaviour of *Rhipicephalus* ticks ("ambushers"), all the inoculation methods were appropriate for screening of entomopathogenic fungal isolates in the pure oil carrier against adults and nymphs. However, while conidia can be applied by direct spraying and ticks retained within the assay arena (SS), the unacceptable high levels of control mortality obtained ruled this method out for routine work. Therefore, based on tick mortality in control and fungus-treated ticks, direct spray treatment followed by removal of ticks to a clean container (SP) is thus a more suitable method for screening entomopathogenic fungi in an oil carrier against adult and nymph and indirect exposure to treated substrate (SW) is suitable for immature stages. The main setback with the indirect inoculation method (SS) is the difficulty to ensure presentation of a precise dose, which will reduce variability in the number of propagules picked by the individual tick (Butt and Goettel 2000). However, free living immature stages of *R. pulchellus* prefer questing from grass in nature and instictively climbed up to the fungus-treated surfaces few minutes after treatment as observed in this study. Furthermore, the lowest tick mortalities in control treatments were recorded following indirect spray (SW).

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