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Performance of a *Metarhizium anisopliae*-treated semiochemical-baited trap in reducing *Amblyomma variegatum* populations in the field

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ABSTRACT

Experiments were carried out to evaluate the efficacy of *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales)-treated semiochemical-baited traps for control of *Amblyomma variegatum* Fabriscius (Acari: Ixodidae) under field conditions. Unfed *A. variegatum* adults (118) were seeded in each 100-m plot and allowed to acclimatise for 3 days. On the fourth day (Day 4), an emulsifiable formulation of *M. anisopliae* (consisting of 49.5% sterile distilled water, fungal conidia, 49.5% corn oil and 1% Tween 80) titrated at 10^9 conidia ml^{-1} was applied in semiochemical-baited traps (900 cm^2) which were placed at five spots within the plot. The control and fungal treatments were repeated after 14 and 28 days soon after rotating the traps clockwise (45°) in order to cover different sections of the plot. In the control plots, emulsifiable formulation without fungus was applied in the semiochemical-baited traps. Six weeks after the initiation of the experiments, five semiochemical-baited traps (untreated) were deployed in each plot for 3 successive days to trap ticks in the treated and control plots. The percentage of ticks recovered in the fungus-treated plots were significantly lower ($31.1 \pm 5.2\%$) than in the control plots ($85.6 \pm 3\%$) ($P < 0.001$), which represented a relative tick reduction of 63.7%. Mortality of $93.8 \pm 2.3\%$ was observed among the ticks that were recovered from the field and maintained in the laboratory for 2 weeks; while only $3.3 \pm 0.9\%$ died from the control plots. The results of this study open up the possibility of developing an environmentally friendly and low cost application strategy to control *Amblyomma* ticks.

1. Introduction

Tick and tick-borne diseases have impacted negatively on development of the livestock industry in Africa (Walker et al., 2003). Ixodid ticks such as *Amblyomma variegatum* Fabriscius and *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae) in particular, are among the most economically important parasites in the tropics and subtropics (Bram, 1983). Control of this pest largely depends on synthetic acaricides (Davey et al., 1998; George et al., 2004) and this has led to heightened concerns over health and environmental impact (Dipeolu and Ndungu, 1991; Gassner et al., 1997). Furthermore, synthetic acaricides are expensive to livestock farmers in Africa who mainly practice subsistence farming. Currently, there is particular interest in microbial control agents, especially entomopathogenic fungi (Maniania et al., 2007).

Inundative and augmentative releases are widely used methods for the introduction of entomopathogens into an ecosystem for control of arthropod pests including ticks (Lacey and Goettel, 1995). For example, Kaaya (2000) and Benjamin et al. (2002) were able to reduce populations of *R. appendiculatus* Neumann (Acari: Ixodidae) and *Ixodes scapularis* Say (Acari: Ixodidae), respectively, following spray application of aqueous formulations of *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales)

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onto the vegetation. Alonso-Díaz et al. (2007) reduced the number of feeding *R. microplus* ticks after spraying naturally infested cattle with an aqueous formulation of *M. anisopliae*. However, there are challenges to the direct application of biopesticides to control ticks on cattle; temperature and host secretions (e.g. sweat), may affect the virulence of entomopathogenic fungi (Polar et al., 2005). Blanket spray of the vegetation may also affect non-target organisms (Hajek and Geottel, 2000; Brownbridge and Glare, 2007) and are expensive requiring significant quantities of materials to treat large areas. Furthermore, some species of ticks such as members of the genus *Amblyomma* (Acari: Ixodidae), which are also known as "hunter" ticks, actively seek their hosts by crawling on soil below the vegetation and may not come into contact with an entomopathogen during blanket spraying of vegetation. Alternative, target methods of applying fungal pathogens to the environment to control ticks are needed. There are opportunities to use autodissemination device to deliver pathogens to ticks (Maniania et al., 2007). Such devices use visual cues, pheromones and kairomones to attract host pests to a pathogen source (Vega et al., 2000). Using a fungus-treated pheromone-baited trap, Maranga et al. (2006) was able to attract and infect *A. variegatum* Fabriscius (Acari: Ixodidae) under field conditions.

Previously, performance of the trap described by Bryson et al. (2000) baited with the semiochemicals (attraction–aggregation–attachment pheromone [AAAP] and 1-octen-3-ol) and treated with the fungus *M. anisopliae* in semi-field experiment was evaluated by Nchu et al. (2009). Ticks attracted to the trap were infected and killed by the fungus, with a subsequent reduction in the tick population. Here, large-scale field trials to evaluate the performance of the fungus-treated trap baited with both the pheromone and kairomone blends against adult of *A. variegatum* populations are described.

2. Materials and methods

2.1. Field site

The experiment was carried out at the National Veterinary Research Centre, Muguga-Kenya Agriculture Research Institute (KARI): latitude 1°13′S, longitude 36°18′E, altitude 2070 m (Obiri et al., 1994). The climate is a modified equatorial type with a mean monthly temperature of 18 °C. Mean annual rainfall is 1005 mm distributed bi-modally with peaks in April and October (Obiri et al., 1994).

The study area was a 0.81-ha paddock and the vegetation was predominantly red oat grass, *Themeda triandra* (Liliopsida: Poaceae). The height of the grass was maintained between 5 and 10 cm. The experiment was done during the rainy season; from April to May of 2008. This period has been associated with high abundance of *A. variegatum* (Walker et al., 2003). The climatic conditions were as follows: mean maximum temperature, 22.75 °C; mean minimum temperature, 13.84 °C; 86.5% RH at 6 a.m.; 57.3% RH at noon; and 177.2 mm rainfall (Department of Meteorology, Kenya). One week prior to the start of the experiment, semiochemical-baited traps were used for sampling of *A. variegatum* in the paddock. No *A. variegatum* tick was found in the plot. However, a few *R. appendiculatus* were attracted to the traps. The field was divided into six plots of 100 m^2 (10 m × 10 m) each, of which three served as controls and three were assigned to fungal treatments. Plots were allocated to both treatments randomly. Plots were separated from each other by a 40 m buffer zone.

2.2. Ticks

Engorged *A. variegatum* females collected from infested cattle from the Marsabit area of Kenya in 2006 were used to established tick colony. Ticks were reared at the Animal Rearing and Quarantine Unit, *icipe*. All life stages of the tick were fed on New Zealand white rabbits. The different instars were maintained in Perspex chambers at 26 °C ± 1 and 85 ± 5% RH under 12:12-h L:D photoperiod. Three to four-week-old unfed adults were used, in the trials.

2.3. Fungus

The *M. anisopliae* isolate (R1/RA), accession number ICIPE 07 used in this study was obtained from the *icipe*'s Arthropod Germplasm Centre. The strain was isolated from an engorged female *A. variegatum* collected from Rusinga Island, Kenya in 1996 and was previously reported to be virulent against *A. variegatum* (Kaaya et al., 1996). The virulence of the fungal strain was maintained by a single passage through the *A. variegatum* (Schaerffenberg, 1964) before being used in the experiment. Conidia were mass produced using long rice as a substrate. Blastospores were cultured in liquid medium containing glucose (30 g/l), peptone (10 g/l) and yeast extract (30 g/l) in a 250 ml Erlenmeyer flask maintained in a shaker at 100 rpm and 26 ± 2 °C for 3 days. Glucose, peptone and yeast extract were obtained from Sigma. The contents of the flask were autoclaved for 30 min at 121 °C and allowed to cool before inoculation with the conidia. Two kilograms of rice per plastic bag was autoclaved for 1 h at 121 °C, transferred to polyethylene autoclavable bags and inoculated with the 3-day old culture of blastospores (50 ml) (Milner R.J., unpubl.). The blastospores suspension was thoroughly mixed with the rice to distribute the inoculum throughout the substrate and incubated between 26 and 30 °C and 60–75% RH for 21 days. The polyethylene bag was then opened and the culture was allowed to dry for 5 days at room temperature to approximately 10–15% moisture content. Conidia were harvested by sifting the substrate through a sieve (295 μm mesh size) and approximately 200 g of spores was produced per bag. Dry conidia were stored in a refrigerator (4–6 °C) for 1–2 weeks prior to use. The viability of conidia was determined using the technique described by Goettel and Inglis (1997) before being used in the field trials. Germination rates >90% after 24 h on Sabouraud dextrose agar was considered adequate for use in the field trials. One litre of emulsifiable conidial suspension containing 1×10^9 conidia ml^{-1} was prepared for the trials (consisting of 49.5% sterile distilled water, fungal conidia, 49.5% corn oil [CHEF cooking oil, Premier Oil Mills Ltd.] and 1% Tween 80). One litre of a control solution was also prepared in a similar manner without the fungus for use in the control plots.

2.4. Semiochemicals and traps

The synthetic components of the attraction–aggregation–attachment pheromone (ortho-nitrophenol, methyl salicylate and nonanioc acid), dichloromethane (DCM) and 1-octen-3-ol were obtained from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Dry ice, which was used as a source of CO_2 was obtained from Carbacid, Kenya. The semiochemical-baited trap used in this study was similar to the one described by Nchu et al. (2009). Briefly, the trap consisted of a 900 cm^2 area demarked by four 10-cm wooden pegs hammered into the ground. A 2 cm × 2 cm × 2 cm rubber sponge impregnated with 0.016 μg of 1-octen-3-ol and 22 μg of AAAP was attached on the top of each of the four wooden pegs per trap and dry ice (CO_2) dispenser (plastic cup) placed in the centre.

2.5. Treatments

Three days before application of the treatments, 118 laboratory-reared adult *A. variegatum* (59 males and 59 females) were seeded in the vegetation in each plot and allowed to acclimatize. Five semiochemical-baited traps were placed at different positions within each plot (one central trap and four diagonal opposed traps, Fig. 1) on the fourth day (day 4) after release of ticks in field plots. The positions of the diagonally opposed traps were moved to new positions by rotating clockwise (45°) while the centrally placed trap was shifted 1 m to the north after 14 days (day 18) (Fig. 1). The positions of the four diagonally opposed traps were moved again after 2 weeks (day 32), moving them 1 m (towards the centre) and clockwise direction by 45°; the central trap was moved 2 m to the south. The area within the confines of each trap (area of trap was 900 cm^2) in the fungal test plots were treated with 250 ml of emulsifiable conidial suspension using a high volume HV applicator (1.5 l model) at a rate of approximately 150 l/ha prior to the attachment of the sponges and impregnation of the sponges with the synthetic semiochemicals at days 0, 18 and 32. All treatments were applied to previously untreated grass immediately after rotating the trap as described above. In the control plots, traps were treated with the emulsifiable carrier without the fungus. The experiment lasted for 6 weeks.

2.6. Evaluation of the efficacy of treatments

At 6 weeks post-treatment (day 46, 47 and 48), five semiochemical-baited traps were deployed in each plot in the morning hours (9.00–12.00 a.m.) in order to attract surviving ticks from the vegetation within the plots. The positions of the traps were also changed daily and collections were made over three consecutive mornings. Ticks collected in each plot were transferred to labelled 9-cm diameter plastic Petri dishes (at most 10 ticks per dish) and brought to the laboratory where they were maintained at 26 ± 1 °C, 85 ± 5% RH and 12:12-h L:D photoperiod for 2 weeks. Mortality was recorded after 2 weeks. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, rinsed twice in sterile distilled water, and then placed into 9-cm diameter Petri dishes lined with moistened filter paper to promote outgrowth of fungi from cadaver to confirm death due to mycosis.

2.7. Fungal persistence

The persistence of inoculum on the treated grass was also investigated during the trial. One uppermost flag leaf of grass that was directly exposed to sunlight was cut within each fungus-treated trap immediately and 2 weeks after treatment using a pair of sterile scissors. Grass samples were kept separately in 9-cm diameter Petri dishes before being transferred to universal bottles containing 10 ml of 0.05% Triton X-100. The samples were shakened vigorously on a vortex shaker for 5 min to dislodged conidia from the treated foliage. The concentration of the fungal suspensions was determined using an

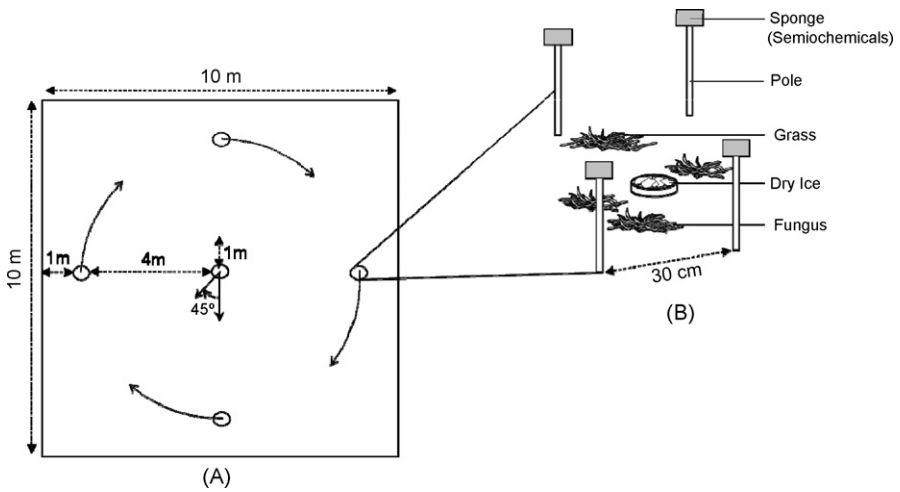

Fig. 1. Schematic diagram showing positions of semiochemical-baited traps in a field plot (A). The trap consist of a 2-cm^2 rubber sponge containing 0.016 μg of 1-octen-3-ol and 22 μg of AAAP fixed on the top of each of four wooden pegs per trap and emulsifiable fungus suspensions sprayed on the grass within each trap (B).

Table 1
The number of *Amblyomma variegatum* ticks recovered from plots containing control and fungus-treated traps, 6 weeks post-treatment and mortality of recovered ticks from control and fungus-treated plots that were maintained under laboratory conditions for 2 weeks.

Treatment	Replicates	Number of ticks recovered from plots, 6 weeks post-treatment	Number of field retrieved ticks that died 2 weeks later under laboratory conditions
Control	Plot 1	98	5
	Plot 2	108	3
	Plot 3	97	2
Test	Plot 1	39	35
	Plot 2	46	44
	Plot 3	25	24

improved Neubauer haemocytometer and was diluted to a concentration of 1.0×10^6 conidia ml^{-1}; 100 μl of the suspension was spread over a SDA plate and a sterile microscope cover slip placed on each plate. Plates were incubated at 25 ± 2 °C, and germination was determined after 24 h, by counting 100 conidia/plate (Goettel and Inglis, 1997). No *M. anisopliae* conidia were recovered from flag leaf collected from control traps, hence the uninoculated control treatment were excluded from the analyses of conidia persistence. Five replicates representing five traps in each plot were used.

2.8. Data analysis

A student's *t*-test was used to compare the following arcsin square root-transformed data at $P = 0.05$ significance level: (i) percentage of ticks recovered from control and fungus-treated plots; (ii) percentage tick mortality in the laboratory of ticks recovered from control and fungus-treated plots; and (iii) percentage germination of conidia recovered from treated foliage, 0 and 14 days post-spray. All analyses were performed using the SAS (2001) package. The relative (%) reduction of tick populations in treated plots was calculated using the formula [(number of surviving ticks recovered from control plots – number of surviving ticks recovered from fungus-treated plots)/ number of surviving ticks recovered from control plots] × 100 (European Medicines Agency, 2004).

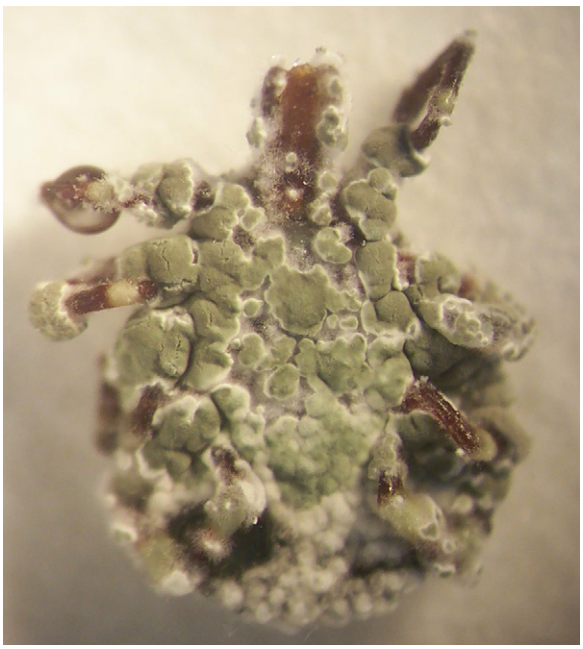

Fig. 2. *Metarhizium anisopliae* conidia developing on *Amblyomma variegatum* cadaver; viewed from the ventral side. Symptoms developed when ticks were held under high conditions of humidity after collection from the field.

3. Results

More ticks were recovered in the control plots compared to the fungus-treated plots at six weeks post-treatment (Table 1). The mean percentage of ticks recovered after 6 weeks in the fungus-treated plots ($31.1 \pm 5.2\%$) was significantly lower ($F = 66.48$; df = 1,4; $P = 0.01231$) than the proportion recovered as a result of fungal activity from the control plots ($85.6 \pm 3\%$), representing a relative reduction of 63.7%. Mortality of ticks that were collected from the field plots after 6 weeks was significantly higher ($F = 586.32$; df = 1,4; $P < 0.0001$) among those collected from fungus-treated plots than the controls (Table 1). Ninety-four (94%) percent of the ticks recovered from the fungus-treated plots succumbed to infection compared to 3% in the controls. The germination of conidia washed from treated foliage was $96.1 \pm 0.41\%$ immediately after spraying but had decreased to $69.2 \pm 1.9\%$ after 14 days, representing a reduction of 28%. All dead ticks among those recovered from fungus-treated plots were covered with conidia but no fungal growth was observed on dead ticks collected from control plots (Fig. 2).

4. Discussion

The high number of ticks recovered from the control plots (85.6%) compared to the fungus-treated plots (31.1%) demonstrates the efficacy of the semiochemical-baited trap. Maranga et al. (2006) reported similar results (76–84% of *A. variegatum* ticks were recovered in the control plots compared to 33.8% in *B. bassiana* and *M. anisopliae*-treated plots) using a relatively more expensive pheromone-baited trap made of aluminium material in comparison with the wooden pegs used for construction of the trap in the current study. While the semiochemical blend used in the present study were attractive to *A. variegatum*, it may not represent the full complement of tick and host odour constituents that *A. variegatum*

requires to locate its preferred hosts. Further studies might reveal additional components, the use of which might enhance the performance of fungus-baited traps. A major advantage of using semiochemicals to attract ticks is that they might facilitate rapid contamination of *Amblyomma* ticks with the fungus used. Most of the ticks that survived infection in the field and brought to the laboratory succumbed to fungal infection, indicating that further mortality may occur beyond the 6 weeks experimental period.

The viability of conidia decreased by 28% at 2 weeks post-treatment in the present study. A variety of environmental factors may influence the viability of entomopathogenic fungi (Ignoffo, 1992). However, it has been demonstrated that an oil-based carrier can minimize the negative effects of environmental stress on fungal conidia compared to aqueous carriers (Inglis et al., 1995). Addition of potential sunscreens and antioxidants to formulations could further increase persistence (Moore et al., 1993), although sunscreens do not appear to promote persistence by a significant amount and the extra cost does not seem to justify their inclusion (Hunt et al., 1994).

In conclusion, this study demonstrates that fungus-treated semiochemical-baited traps can control *A. variegatum* under field conditions. This strategy could be valuable for reducing *Amblyomma* population around water points, camping sites, salt licks and paddock facilities. Such traps could also be used to control other *Amblyomma* species and other "hunter" ticks. The use of semiochemicals (tick pheromones and host kairomones) and entomopathogenic fungi in a trap has demonstrated potential for tick control off-host. This has the additional advantage in that it reduces the risks of transmission of tick-borne diseases by killing ticks before they become attached to a host. Furthermore, this technology would minimize the area treated with a mycoacaricide, thus reducing non-target contamination. Further studies are required for operational strategies to be developed, and to improve trap efficacy. For example, refinements to the formulation may promote conidial persistence, and optimization of attractant components may enhance field performance.

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