# Evaluating the Efficacy of Oil-Based Entomopathogenic Fungi conidial Formulations on Dog Ticks

**Darrell Nadeng Dominic[1], Jasmine Siah[2], Brenda Chan[2], Peter Morin Nissom[2#]**

1 School of Research, Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, 93350 Kuching, Sarawak, Malaysia.
2 School of Chemical Engineering and Science, Faculty of Engineering, Computing and Science, Swinburne University of Technology Sarawak Campus, 93350 Kuching Sarawak Malaysia.
#Corresponding author. E-Mail: pmorin@swinburne.edu.my; Tel: +60 82 260 939; Fax: +60 82 260 813

**ABSTRACT** *Rhipicephalus sanguineus* and *Haemaphysalis longicornis* are common dog ticks in Malaysia and they act as vectors to pathogens which spreads onto animal hosts and, in some cases, humans. Common control strategies employ synthetic chemical products which are not sustainable as it contributes to the rise of insecticide resistance. The use of entomopathogenic fungi as a mycological based insecticide has been widely researched as a potential alternative strategy against pest insects. Entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have been reported to kill a range of pest-insects including ticks in the lab. In this research, we evaluated the efficacy of sunflower oil-formulated spray, and its efficiency to induce mortality in different stages of dog ticks. Our findings reports 20% sunflower oil formulations containing x$10^8$ *M.anisopliae* spores/ml induces death within an average of 2.67±1.49 days in engorged female adult ticks and 3.00±0.63 days in tick larvae, while *B.bassiana* spores at the same concentration induces death within an average of 3.21±1.13 days in engorged female adult ticks and1.67±0.52 days in tick larvae. Our findings reported tick larvae to be more susceptible to *B.bassiana* spores formulated with 20% sunflower oil as compared to the engorged female adult ticks. The results in the present study will be potentially beneficial for future applications in the field.

**KEYWORDS:** Entomopathogenic Fungi; *Metarhizium anisopliae*; *Beauveria bassiana*; Biological Control; Dog Ticks

**I Received** 23 January 2019 **II Revised** 21 February 2019 **II Accepted** 28 February 2019 **II Online** 5 March 2019 **II © Transactions on Science and Technology I**

## INTRODUCTION

Ticks are blood-sucking pests that commonly infect the livestock industry (i.e. cattle) and common household pets (i.e. dogs). Ticks act as vectors to various diseases such as Lime disease in humans and are known to undergo a three-host life cycle (Centers for Disease Control and Prevention 2017). This increases the potential for diseases to spread from one host to another at a rapid rate as they exchange hosts after every moulting stage. In the livestock industry, ticks are almost certainly present, making the industry a reservoir for ticks to breed. They act as parasites to animals such as cows, and chronic infestations could constitute devastating effects on the health of the host, indirectly affecting the quality of produce from the farm (Jongejan & Uilenberg, 2004; Eskezia, 2011). Hence, the imperative need for pest-control in the livestock industry to maintain the quality of produce to prevent losses. Current control strategies employ the use of synthetic pesticides such as acaricides for the mitigation of ticks. Synthetic pesticides are still the main choice for pest-control due to its low-purchase price and its efficiency to produce desirable results. However, the major drawbacks from the use and abuse of such chemicals have recently manifested itself as a bigger threat to mankind and the environment.

Acaricides and synthetic pesticides alike often contains poisonous active ingredients such as organophosphates and benzene hexachloride (Coles & Dryden, 2014) which ticks are starting to develop resistance to overtime. Biological control employs the use of biocontrol agents such as microbes for the mitigation of pests. The integration of such bio-based technology is known to be environmentally friendly and sustainable in a long run (Brodeur, 2012). Entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* are two well-known entomopathogenic funguses researched due to its effectiveness to kill various insects such as rhinoceros beetles in the agricultural industry. Existing studies venture into synergistic combinations utilizing carriers such



TRANSACTIONS ON SCIENCE AND TECHNOLOGY

as solvents and oils to further improve the ability of the conidia to survive in the field (Hedimbi *et al.*, 2011; Kaaya *et al.*, 2011). Barreto *et al.*, (2016) reported improved survival rates and protection against harsh conditions in the field when fungal conidia are formulated with oil formulations. In a similar study, Camargo *et al.*, (2012) reported mineral oil formulations efficient in controlling *Rhipicephalus micropus* ticks as oppose to aqueous-based formulations in the lab. This initiated our interest to explore the effectiveness and efficiency of oil-based formulations for pest control. With the aim of evaluating the acaricidal properties of sunflower oil for the control of dog ticks, preliminary experiments were first carried out to evaluate the interaction of the fungi in sunflower oil. A bioassay was then carried out using 20% sunflower oil formulation was performed on different stages of dog ticks to determine its efficiency to induce death. Results in this study will be potentially beneficial for future field applications.

## METHODOLOGY

*Collection of Dog Ticks*

Healthy Engorged Female (EF) adult dog ticks and larvae were collected from pet dogs in Kuching, Sarawak. EF adult ticks were targeted as they are responsible for oviposition, and freshly hatched larvae from eggs collected were used instantly to evaluate efficacy. The species of dog ticks were identified based on key identification traits. *Rhipicephalus sanguineus* are identified by their hexagonal basis capitula while *Haemaphysalis longicornis* has a Y-shaped anal groove embracing its anus posteriorly (Zheng *et al.*, 2011). Tick larvae were identified based on smaller size, translucent body, and number of legs whereby larvae have 3 legs on each side.

*Fungal Cultures and Production of Conidia*

*Metarhizium anisopliae* (ATCC™ 9454) and *Beauveria bassiana* (ATCC™ 26852) were maintained on Potato Dextrose Agar (HiMedia, India) and incubated in 25°C for 2 weeks. Production of conidial spores were performed using Solid State Fermentation (SSF) in rice (11g of rice + 20ml dH2O supplemented with 1% yeast extract) in a 250mL Erlenmeyer flask and sealed tightly with non-absorbent cotton wool. Five fungi agar plugs were transferred aseptically into the solid media using sterile straws. Flasks were incubated at 25°C for 2 - 3 weeks until sporulation. Conidia were harvested by flooding the flask with 0.05% Tween 80 (Fisher Scientific, USA) and agitated on an orbital shaker (Cole-Parmer, USA) for 30 minutes at 250 rpm. The contents were filtered through a muslin cheese cloth to remove larger debris and hyphae. Filtered spore solutions collected were then washed by centrifugation with 0.05% Tween 80 and stored in 4°C until use. Sunflower oil at a concentration of 20% was prepared and mixed with $x10^8$ spores/mL for the assays.

*Preliminary Assessment on the Fungal Conidia Germination and Mycelia Growth*

The conidia germination assay was adapted from Ummidi and Vadlamani, (2014) with slight modifications by which only qualitative observation was performed to observe for signs of positive germination. 50µL of oil formulation at $10^8$ conidia/mL was pipetted onto water agar and one sterile cover slip was placed directly on top of the formulation droplet. The plates were sealed and incubated for 24 hours at 25°C. After 24 hours, the cover slip was carefully removed from the agar and observed under the microscope for signs of conidial germination. Mycelial growth assessment was carried out by first removing fungal agar plugs using sterile straws from 2-week-old cultures on PDA and dipping them into the different concentrations of sunflower oil. The agar plugs were allowed to dry before transferring one agar plug onto a PDA plate. Three replicates were observed for each concentration. The plates were incubated for 14 days at 25°C and the diameter was measured at the end of the incubation period.

*Bioassay on Dog Ticks*

Ticks obtained were placed in assay plates made of disposable Petri dishes lined with moist filter paper. Each assay plate contains one dog tick and sprayed once with the formulation using a 100mL spray bottle (DAISO, Japan). Ten replicates were maintained in each group (MA group, BB group, and Control group) for EF adult tick assay, while 5 replicates were maintained for the tick larvae assay as the number of instantly hatched larvae was lesser. Control group was sprayed with 20% sunflower oil containing 0.05% tween 80 only. Assay plates were closed loosely with the Petri dish lid and were maintained in a humidity chamber controlled at 22±2°C and >90% relative humidity. Observations were conducted daily with the aid of a stereo binocular microscope, and ticks were labelled as dead once they are seen immobile.

*Statistical Analysis*

The significance of the data obtained from the bioassay on EF adult and larvae dog ticks were analyzed by one-way analysis of variance ($P<0.05$) using SigmaPlot 14 statistical software. The significance of the treated groups in each stage was compared to the control group using Dunnett's Test and the data was expressed as mean of ten replicates for time (days) for the ticks to die ± standard deviation and mean of five replicates for time (days) for the ticks to die ± standard deviation for the tick larvae assay. The significance of the same treatment for different stages was analyzed by student t-test *($P<0.05$)*

## RESULT AND DISCUSSION

*Preliminary Assessment on the Fungal Conidia Germination and Mycelia Growth*

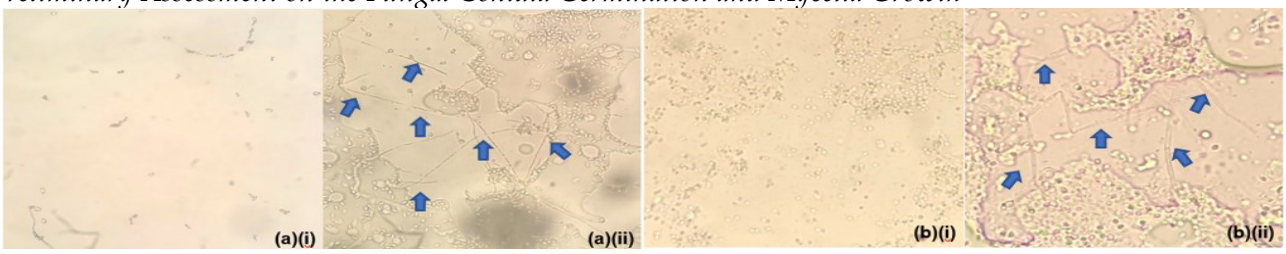


**Figure 1.** Microscopic observations on the germination of (a) *M. anisopliae* spores after 24 hours; (i) germination of spores in 0% oil, (ii) germination of spores in 20% oil, and (b) *B. bassiana* spores after 24 hours; (i) germination of spores in 0% oil, (ii) germination of spores in 20% oil.

Positive germination was noted as the presence and elongation of germ tubes which included swelling of spores after incubation (Moore *et al.*, 2000). Microscopic observations after 24 hours incubation showed long germ tubes present in spores exposed to all the concentrations of sunflower oil formulations. This was compared to the 0% sunflower oil concentration for both *M.anisopliae* and *B. bassiana*. Germ tubes present were indicated by arrows on Figure 1. An existing study reported sunflower oil to be incompatible and had toxic effect towards *B. bassiana* (Ummidi & Vadlamani, 2014). Our observations however show germinations of *B. bassiana* in sunflower oil possible. The mycelia growth of each fungus exposed to different concentrations of sunflower oil were determined by measuring the diameter of the mycelia growth (Table 1). Results obtained showed diameter of both fungi exposed to the highest concentration of sunflower oil to have no significant difference ($P<0.05$) as compared to the lower concentrations, except for *B.bassiana* in 0% sunflower oil. This provided a good indication that the sunflower oil doesn't exhibit any toxic properties to the growth of the fungi even at lower concentrations and without oil. As 20% oil was observed to homogenize well in the spore solutions containing 0.05% tween 80, 20% sunflower oil was used for the bioassay.

**Table 1.** Growth diameter of *M.anisopliae* and *B.bassiana* mycelia agar plugs immersed in different sunflower oil concentrations. Data was expressed as mean diameter (cm) ± standard deviation.

| Oil concentration | Mean Diameter of Mycelia, cm | |
|---|---|---|
| | ***M. anisopliae*** | ***B. bassiana*** |
| 0% | 5.30±1.06 | 3.50±0.50 |
| 5% | 5.40±1.05 | 4.27±0.50 |
| 10% | 5.40±0.50 | 4.37±0.06 |
| 15% | 5.80±0.26 | 4.07±0.50 |
| 20% | 5.43±0.51 | 4.53±0.15 |

*Bioassay on Dog Ticks*

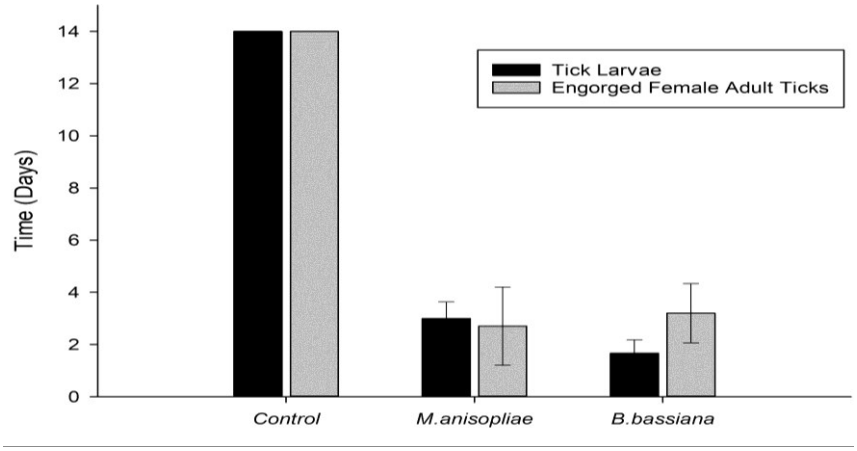


**Figure 2.** Duration for death post-exposure to entomopathogenic fungi formulations on different stages of dog ticks for a period of 14-days. Note: Results are means of ten replicates for adults and means of five replicates for larvae. Error bars represent standard deviation.

The time taken for 20% sunflower oil formulation to induce death on the ticks was measured in this study to determine its efficiency. Daily microscopic observations were performed to determine whether the ticks were dead, and their mortality was confirmed once they were seen immobile. Our findings indicated 20% sunflower oil formulations of both *M.anisopliae* and *B.bassiana* conidia to have successfully induced mortality within an average of 2-4 days after exposure in both stages of dog ticks (Figure 2). The obtained results were found to be statistically significant *(P<0.05)* through a single factor ANOVA test for each stage. A post hoc test was conducted with Dunnett's Test indicated the treatment groups were statistically significant in both EF adult stage and larvae stage when compared to its respective control group *(P<0.001)*. EF adult ticks treated with formulated *M. anisopliae* spores died faster on an average of 2.67±1.49 days while ticks in the larvae stage were observed to achieve mortality on an average of 3.00±0.63 days. In contrast to the adult stage, 20% sunflower oil formulated *B. bassiana* spores killed EF adult ticks within an average of 3.20±1.14 days, slower than the tick larvae which died within an average of 1.67±0.52 days after exposure. Samish *et al*., (2001) reported similar findings in their larvicial assay against *R. sanguineus* larvae whereby deaths were observed between 2 to 3 days after exposure to fungi. However, they did not integrate any use of oil formulations. Our results suggested tick larvae to be more susceptible to *B.bassiana* in the formulated 20% sunflower oil as compared to the EF adult ticks *(P<0.05)*. This contrasts the findings by Samish *et al*., (2001) where they reported *M.anisopliae* to be more potent, however using a different strain of *M.anisopliae* fungus. The data for EF adult tick and larvae treated with *M.anisopliae* formulations were not found to be statistically significant *(P<0.05)*.

*Microscopic Observations*

Microscopic observations on EF adult ticks showed heavy presence of fungi mycelia on the cadavers of the treated groups on the 8th day while the control group remained active and alive (Figure 2). The mortality of the EF tick was observed to not possess noticeable mycelial growth on its cuticle prior to death but was only observed to have fungal growth within 3-4 days after death, similar to the description of Samish *et al*., (2001). Samish *et al*., (2001) described fungal infection on adult ticks as the appearance of hemorrhages from below the cuticle which leads to the appearance of blood escaping the cuticles. Our study reports red blister-like appearances on the cuticle of the treated EF adult ticks within 2-3 days post exposure to the fungal formulations. This follows by immobility and death in the EF adult ticks after exposure to fungal formulations. We believe the production of cuticle-degrading enzymes and mycotoxins in the fungal formulations played a significant role in the pathogenicity of the fungal formulations. Microscopic observations on the tick larvae showed dark lumps protruding on the surface of the dead tick exposed to formulations with *M.anisopliae* (Figure 3(a)). Larvae exposed to the formulations with fungi were observed to be lethargically immobile after 24hours, similar to the description of infection by Samish *et al*., (2001). This contrasts with the control group which appears to be mobile and well.

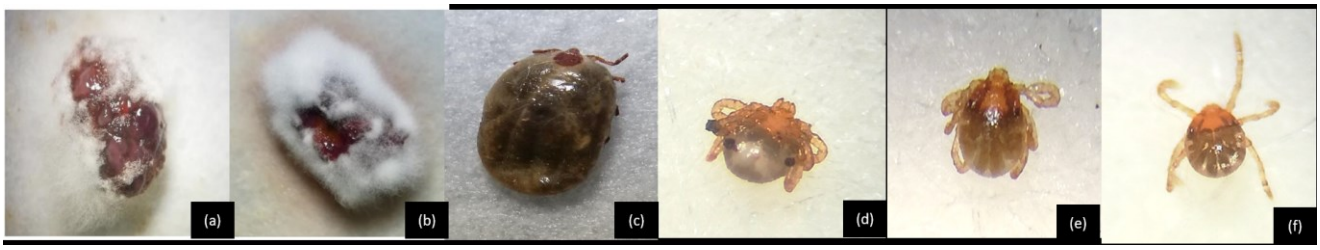


**Figure 3.** Microscopic image on EF adult ticks on the 8th day of the bioassay (a) Cadaver from *M.anisopliae* cluster, (b) Cadaver from *B.bassiana* cluster, and (c) EF adult tick from control cluster; and microscopic image on tick larvae on the 5th day (a) Cadaver from *M.anisopliae* cluster, (b) cadaver from *B.bassiana* cluster, and (c) Tick larvae from the control cluster. **Note:** Red blistering can be observed on the cadavers of both EF adult tick treated groups, while dark lumps are present on the tick larvae from *M.anisopliae* cluster.

Utilizing the sunflower oil formulation at a higher concentration increases the adhesion of spores to the surface of the tick's cuticles which would be very beneficial in a field setting Additionally, oil formulations protect the spores from UV radiations by the sunlight (Hedimbi *et al*., 2008). This encourages the application of oil formulations in a field setting. Ticks are incapable of drinking water, thus the requirement of an environment with high humidity (≥85%) therefore the ticks were kept in a humidity chamber (≥90%) throughout the bioassay period. Ticks that laid eggs during the bioassay period were disqualified from the assay and replaced *(Kurlovs et al*., 2014). These parameters help reduce the incidence of premature deaths which will compromise the reliability of the data.

## CONCLUSION

In summary, it has been demonstrated through this study that 20% sunflower oil-based fungal formulations with *M.anisopliae* and *B.bassiana* kills both EF adult and tick larvae effectively within an average of 2-4 days in the lab. 20% sunflower oil formulations with *B. bassiana* was observed to be more potent against larvae stages as compared to the EF adult stage, and our preliminary studies indicated sunflower oil at concentrations up to 20% to have no toxic properties against the germination of spores and growth of mycelia. Through these findings, we aim to apply these oil formulations under field conditions in the future.

## ACKNOWLEDGEMENTS

The authors would like to express their sincere appreciation to the Malaysian Ministry of Energy, Science, Technology, Environment and Climate Change for partially funding this research (Grant no. FP0514D0025-2(DSTIN)).

## REFERENCES

[1] Barreto, L. P., Luz, C., Mascarin, G. M., Roberts, D. W., Arruda, W. & Fernandes, É. K. K. (2016). Effect of Heat Stress and Oil Formulation on Conidial Germination of *Metarhizium anisopliae s.s.* on Tick Cuticle and Artificial Medium. *Journal of Invertebrate Pathology*, **138**, 94-103.

[2] Brodeur, J. (2012). Host Specificity in Biological Control: Insights from Opportunistic Pathogens. *Evolutionary applications*, **5**(5), 470–480

[3] Camargo, M. G., Golo, P. S., Angelo, I. C., Perinotto, W. M. S., Sá, F. A., Quinelato, S. & Bittencourt, V. R. E. P. (2012). Effect of Oil-Based Formulations of Acaripathogenic Fungi to Control *Rhipicephalus microplus* Ticks Under Laboratory Conditions. *Veterinary Parasitology*, **188**(1), 140–147.

[4] Centers for Disease Control and Prevention (CDC) (2017). *Ticks* (https://www.cdc.gov/dpdx/ticks/index.html), Last accessed on 18 January, 2019.

[5] Coles, T. B. & Dryden, M. W. (2014). Insecticide/Acaricide Resistance in Fleas and Ticks Infesting Dogs and C ats, *Parasites & Vectors*, **7**(1), 9-10.

[6] Eskezia, B. G. (2011). Review on the impact of ticks on livestock health and productivity. *Journal of Biology, Agriculture and Healthcare*, *Journal of Biology, Agriculture and Healthcare*, **6**(22), 1-7.

[7] Hedimbi, M., Kaaya, G. P., Singh, S., Chimwamurombe, P. M., Gindin, G., Glazer, I. & Samish, M. (2008) Protection of *Metarhizium anisopliae* Conidia from Ultra-violet Radiation and their Pathogenicity to *Rhipicephalus evertsi evertsi* Ticks. *Experimental and Applied Acarology*, **46**(1), 149–156.

[8] Hedimbi, M., P Kaaya, G. & Chinsembu, K. (2011). Mortalities Induced by -Entomopathogenic Fungus *Metarhizium anisopliae* to Different Ticks of Economic Importance using Two Formulations. *International Research Journal of Microbiology*, **2**(4), 141-145.

[9] Jongejan, F. & Uilenberg, G. (2004). The Global Importance of Ticks. *Parasitology*, **129**(S1), S3–S14.

[10] Kaaya, G. P., Samish, M., Hedimbi, M., Gindin, G. & Glazer, I. (2011). Control of Tick Populations by Spraying *Metarhizium anisopliae* Conidia on Cattle Under Field Conditions. *Experimental and Applied Acarology*, **55**(3), 273–281.

[11] Kurlovs, A. H., Li, J., Cheng, D. & Zhong, J. (2014).Ixodes Pacificus Ticks Maintain Embryogenesis and Egg Hatching after Antibiotic Treatment of Rickettsia Endosymbiont. *PloS one*, **9**(8), e104815–e104815.

[12] Moore, D., Robson, G. D. & Trinci, A. P. J. (2000) *21st Century Guidebook to Fungi*. Cambridge: Cambridge University Press.

[13] Samish, M., Gindin, G., Alekseev, E. & Glazer, I. (2001) Pathogenicity of Entomopathogenic Fungi to Different Developmental Stages of *Rhipicephalus sanguineus* (Acari: Ixodidae). *The Journal of Parasitology*, **87**(6), 1355-1359.

[14] Ummidi, V. R. S. & Vadlamani, P. (2014) Preparation and Use of Oil Formulations of *Beauveria bassiana* and *Metarhizium anisopliae* against *Spodoptera litura* Larvae. *African Journal of Microbiology Research*, **8**(15), 1638–1644.

[15] Zheng, W., Chen, H., Liu, X., Guo, X. & Fu, R. (2011) Severe Tick Infestation in a Hare and Potential Risk for Transmitting Pathogens to Humans. *Korean J Parasitol*, **49**(4), 419–422.