

*Original Research***In-vitro Evaluation of Entomopathogenic Fungus *Metarhizium anisopliae* against the Cattle Tick *Rhipicephalus (B.) microplus* (Acarina: Ixodidae)****Greeshma Rao, U. B. and Babasaheb Wamanrao Narladkar**

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**Abstract**

The research was conducted to evaluate the pathogenicity of fungus *Metarhizium anisopliae* on the egg and adult stages of the cattle tick *Rhipicephalus (B.) microplus*. The fungal powder containing conidia procured in the year 2012 and manufactured in the year 2016 was used in the research to compare their efficacy against egg and adult stages of *Rhipicephalus (B.) microplus* ticks. The susceptibility of the tick to the species of entomopathogenic fungi was evaluated by treating engorged female ticks and eggs with fungi at concentrations  $1g \times 10^8$  conidia/litre,  $2 \times 10^8$  conidia/litre  $4g \times 10^8$  conidia/litre,  $5g \times 10^8$  conidia/litre  $6g \times 10^8$  conidia/litre. The treatment of ticks and egg was followed as per standard procedure. The both fungal treatments showed at par results in terms of mortality of adult ticks, reduction in egg laying capacity and hatchability of the treated eggs in comparison with the control and found to be concentration dependent. The hatching rates by the treated groups were inversely proportional to the concentration of conidia used. The results showed that the biological control of *R. (B.) microplus* by *M. anisopliae* infection might constitute an additional method to integrated tick management.

**Key words:** Integrated Tick Management, *Metarhizium anisopliae*, *Rhipicephalus (B.) microplus*

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**Introduction**

Ticks are obligatory blood sucking arachnids that feed on vertebrates. While argasid ticks (soft ticks) feed for minutes or hours, the ixodid ticks (hard ticks) feed for days to weeks. The *Rhipicephalus (B.) microplus* is an obligatory haematophagus ectoparasite of terrestrial vertebrates with single host occurs in almost all tropical and subtropical countries and is a major vector for bovine babesiosis and anaplasmosis (Sharma *et al.*, 2013) and also transmits various pathogenic microorganisms such as protozoa, rickettsiae, spirochaetes and viruses (Jongejan and Uilenberg 2004).

In general all tick species transmit a greater variety of infectious organisms than any other group of arthropods and worldwide, are second only to mosquitoes in terms of their public health and veterinary importance (Levin, 2016). *When economic significance of ticks is taken into account it proves that* these ticks have a debilitating action; production losses by acting as a vector to haemoprotozoan diseases, also losses to the tanning industry and dairy industry. Tick control throughout the world is based mainly on the repeated use of chemical acaricides. Unlimited use of this tick controlling measures has resulted in problems related to environmental pollution, milk contamination and resistance development in the target species (Onofre *et al.*, 2001). Thus scientists are concentrating on non-chemical alternatives, one of them is biological control. Biological method of tick control involves the use of entopathogenic fungi. Therefore present study was planned with the objective of evaluating the efficacy of fungal bio-control agent, *Metarhizium anisopliae* against egg and adult stages of *Rhipicephalus microplus* ticks.

## Materials and Methods

The research work was undertaken at Department of Veterinary Parasitology and College of Veterinary and Animal Sciences, (MAFSU) Parbhani, Maharashtra, India.

### Fungal Species Studied in the Experiment-*Metarhizium anisopliae*

#### Procurement of the Fungal Powder

The fungal powder was procured from Biological control unit, Department of Agril Entomology, Mahatma Phule Krishi Vidyapeeth Rahuri Dist Ahemednagar (MS), India. The fungal powder procured five years back (2012) and manufactured in the year 2016 was used in the research to compare their efficacy against various developmental stages of *Rhipicephalus (B.) microplus* ticks.

#### Collection of Ticks

Requisite number of blood engorged female ticks was collected from cattle (cows/bullock) body using forceps at the junction of skin and ticks.

#### Identification

Ticks were identified under zoom stereoscopic microscope as *Rhipicephalus (B.) microplus* before being introduced in the experiment Walker *et al.* (2003).

#### Experimental Design

For all *in vitro* trials, working concentration were prepared as 1,2,4,5 and 6gm powder+ 5ml milk + 1 liter of water. Test procedure described by Bagherwal *et al.* (1994), Maske *et al.* (2000), Kaaya and Hassan (2000) and Narladkar *et al.* (2015) were followed with little modification.

### For Adult Ticks

In each diluted concentration 10 female ticks were dipped for 1 minute, were dried on filter paper and then placed in test tube as single tick per tube, followed by closure of tube with muslin cloth and rubber band. The mortality of ticks was observed at 24 hrs till 96 hrs. The mortality data were tabulated and efficacy was worked out in terms of per cent mortality. If the ticks were not dead, they were judged for their egg laying capacity; observed for number of eggs laid and compared with control ticks which were only treated with water.

### Action on Eggs of Treated Females

The treated female *Rhipicephalus (Boophilus) microplus* ticks not dead, were separately maintained for eggs collection. Eggs collected from such treated female ticks were counted in petri dishes in the batches of 100 numbers, were transferred in tubes which were closed with piece of muslin cloth tied with rubber band. These tubes were maintained in desiccators in which humidity levels were maintained @ 75 %. The eggs were observed for hatching, till the period hatching process of eggs in the control group were completed.

### On Treated Eggs

The eggs laid by *Rhipicephalus (B.) microplus* ticks in the tubes separately maintained for egg collection, were drawn from tubes in petri dishes. By counting the eggs in 100 numbers were treated with different fungal concentration solution, dried on filter paper and then transferred in tubes. These tubes were maintained in desiccators in which humidity levels were maintained @ 75 %. The eggs were observed for hatching, till the period hatching process of eggs in the control group were completed.

### Criteria's for Assessment of Efficacy of *Metarhizium anisopliae*

- a. Mortality of adult ticks and eggs
- b. Reduction in egg laying capacity
- c. Hatchability of eggs from treated females
- c. Hatchability of treated eggs

### Statistical Analysis

The data obtained from various parameters were analyzed by employing two factor Factorial Experiment using computer applications, WASP.

### Results and Discussion

The average mortality, egg laying capacity, hatchability of eggs from treated female ticks and hatchability of the treated eggs count observed after the treatment with fungi showed variation in the mortality count

at different concentrations and their average count at different concentrations are depicted in the Table 1,2,3,4 respectively.

### On Mortality of Adult Ticks

If you look into numerical values, it appears that amongst 10 ticks treated for each fungus at different concentrations; maximum average mortality of 43% was recorded and minimum of 0 (no ticks dead); indicating and suggesting fungus has effect in causing mortality of adult ticks *in-vitro*, however mortality rate appears to be concentration dependent and with increasing concentration of fungal conidia mortality count was also increased (Table 1).

**Table 1:** Showing Mean mortality of *Rhipicephalus (B) microplus* female ticks after treatment with various fungi at different concentrations

S. No.	Treatments	1g/l Mean±SE Range	2g/l Mean±SE Range	4g/l Mean±SE Range	5g/l Mean±SE Range	6g/l Mean±SE Range	CD
I	<i>Metarhizium anisopliae</i> Rahuri Old	<sup>p</sup> 0.15 <sup>a</sup> ±0.15 (0-1)	<sup>p</sup> 0.29 <sup>b</sup> ±0.18 (0-1)	<sup>p</sup> 0.29 <sup>b</sup> ±0.18 (0-1)	<sup>p</sup> 0.29 <sup>b</sup> ±0.18 (0-1)	<sup>p</sup> 0.43 <sup>c</sup> ±0.20 (0-1)	(5%) 0.12
II	<i>Metarhizium anisopliae</i> Rahuri New	<sup>p</sup> 0.00 <sup>a</sup> ±0.00 (0-0)	<sup>pq</sup> 0.15 <sup>b</sup> ±0.15 (0-1)	<sup>p</sup> 0.29 <sup>c</sup> ±0.18 (0-1)	<sup>p</sup> 0.29 <sup>c</sup> ±0.18 (0-1)	<sup>p</sup> 0.43 <sup>d</sup> ±0.20 (0-1)	
III	Control	<sup>p</sup> 0.00±0.00 (0-0)	<sup>q</sup> 0.00±0.00 (0-0)	<sup>q</sup> 0.00±0.00 (0-0)	<sup>q</sup> 0.00±0.00 (0-0)	<sup>q</sup> 0.00±0.00 (0-0)	
Critical Difference(CD)		(5%)0.167			(1%)0.224		

Superscripts a,b,c,d indicates significant differences between concentrations ( amongst the rows)  
Superscripts p,q significant differences between different fungi (amongst the columns)

In the control group no mortality was recorded. Therefore as an adulticide all the tested fungal strains exerted much expected effect. The results obtained were in agreement to the author Monteiro *et al.*, (1998) who recorded a mortality of 50%.

### Egg Laying Capacity

The treatment with both fungi resulted in significant reduction in egg laying capacity of the female adult ticks compared to control group. If you look into numerical values, it appears that amongst 10 ticks treated for each fungus at different concentrations; maximum reduction in egg laying was recorded for new strain i.e. 23.57eggs while minimum reduction in egg laying was recorded with the treatment of old fungus i.e. 214.29 (Table 2). In contrast to these values, control ticks laid 1578.55 eggs. The result of eggs to an extent of 90% was noted by the author Lopez *et al.*, 2009 on *R. microplus* ticks.

### Hatchability of Eggs from Treated Females

The treatment with both fungi resulted in significant reduction in hatchability of the eggs, which were harvested from the treated female ticks (eggs were not directly exposed to the fungal treatment). Even 10% eggs could not be hatched as against hatchability of 97.7 in control tick eggs. At the concentration

of 6g/l, all the eight fungal treatments showed the effect to their maximum and hatching of eggs could be noted to the lowest as 0.71 to 2.29. These results were in contrast to the author Broglio-Micheletti *et al.*, (2012) who found no reduction in hatchability of *B. microplus* eggs.

**Table 2:** Showing mean egg laying capacity of *Rhipicephalus (B) microplus* female ticks after treatment with various fungi at different concentrations

S. No	Treatments	1g/l Mean±SE Range	2g/l Mean±SERange	4g/l Mean±SERange	5g/l Mean±SERange	6g/l Mean±SERange	CD
I	<i>Metarhizium anisopliae</i> Rahuri Old	<sup>p</sup> 214.29± 76.94 (0-600)	<sup>p</sup> 125.71±53.99 (0-400)	<sup>p</sup> 118.57±69.36 (0-500)	<sup>p</sup> 102.85±53.75 (0-400)	<sup>p</sup> 70.000±55.807 (0-400)	(5%) 80.218
II	<i>Metarhizium anisopliae</i> Rahuri New	<sup>q</sup> 50.71±20.71 (0-150)	<sup>q</sup> 47.43±28.70 (0-200)	<sup>p</sup> 34.85±11.21 (20-100)	<sup>p</sup> 34.29±17.17 (0-100)	<sup>p</sup> 23.57±8.92 (0-60)	
III	Control	<sup>r</sup> 1578.55±261.32 (350-2315)	<sup>q</sup> 1578.55±261.32 (350-2315)	<sup>q</sup> 1578.55±261.32 (350-2315)	<sup>q</sup> 1578.55±261.32 (350-2315)	<sup>q</sup> 1578.55±261.32 (350-2315)	
	Critical Difference(CD)	(5%) 107.621			(1%)141.448		

Superscripts p,q,r indicates significant differences between different fungi (amongst the columns)

**Table 3:** Showing mean hatchability of eggs harvested from *Rhipicephalus (B) microplus* female ticks treated with various fungi at different concentrations

S. No	Treatments	1g/l Mean±SE Range	2g/l Mean±SE Range	4g/l Mean±SE Range	5g/l Mean±SE Range	6g/l Mean±SE Range	CD
I	<i>Metarhizium anisopliae</i> Rahuri Old	<sup>p</sup> 8.57 <sup>a</sup> ±1.78 0-12	<sup>p</sup> 8.15 <sup>a</sup> ±1.84 2-15	<sup>p</sup> 4.00 <sup>b</sup> ±0.81 0-6	<sup>p</sup> 3.29 <sup>bc</sup> ±1.29 0-10	<sup>p</sup> 2.29 <sup>c</sup> ±1.40 0-10	(5%) 1.290
II	<i>Metarhizium anisopliae</i> Rahuri New	<sup>q</sup> 3.85 <sup>a</sup> ±2.30 0-15	<sup>q</sup> 2.15 <sup>b</sup> ±1.36 0-10	<sup>q</sup> 1.43 <sup>bc</sup> ±0.68 0-4	<sup>q</sup> 0.85 <sup>c</sup> ±0.45 0-3	<sup>p</sup> 0.71 <sup>c</sup> ±0.56 0-4	
III	Control	<sup>r</sup> 97.57±0.71 95-100	<sup>r</sup> 97.57±0.71 95-100	<sup>r</sup> 97.57±0.71 95-100	<sup>r</sup> 97.57±0.71 95-100	<sup>q</sup> 97.57±0.71 95-100	
	Critical Difference(CD)	(5%)1.748			(1%)2.295		

Superscripts a,b,c indicates significant differences between concentrations (amongst the rows)

Superscripts p,q,r indicates significant differences between different fungi (amongst the columns)

### Hatchability of Treated Eggs

The data from Table 5 indicates that, at all the concentrations may be for 1g/l, 2g/l, 4g/l, 5g/l or 6g/l for both fungi, the hatchability was significantly reduced with increasing concentration of conidia, as compared to control group eggs and it has achieved its lowest levels at 6g/l concentration. The hatchability of the control group was observed as 97.57. Similar types of results were recorded by Narladkar *et al.*, 2015 who also found significant reduction in hatchability upon treatment with the fungus.

**Table 4:** Showing mean hatchability of the *Rhipicephalus (B) microplus* tick eggs after treatment with various fungi at different concentrations

Sl. No	Treatments	1g/l Mean±SE Range	2g/l Mean±SE Range	4g/l Mean±SE Range	5g/l Mean±SE Range	6g/l Mean±SE Range	CD
I	<i>Metarhizium anisopliae</i> Rahuri Old	<sup>p</sup> 40.60 <sup>a</sup> ±5.13 30-60	<sup>p</sup> 35.83 <sup>b</sup> ±10.83 0-70	<sup>p</sup> 32.00 <sup>b</sup> ±6.04 15-50	<sup>p</sup> 11.80 <sup>c</sup> ±2.20 7-20	<sup>p</sup> 5.60 <sup>d</sup> ±1.50 2-10	(5%) 4.521
II	<i>Metarhizium anisopliae</i> Rahuri New	<sup>q</sup> 89.40 <sup>a</sup> ±2.21 82-96	<sup>q</sup> 82.20 <sup>b</sup> ±2.08 77-89	<sup>q</sup> 66.20 <sup>c</sup> ±3.33 59-77	<sup>q</sup> 38.60 <sup>d</sup> ±4.29 28-52	<sup>q</sup> 9.60 <sup>e</sup> ±1.72 5-15	
III	Control	<sup>r</sup> 97.57±0.86 95-100	<sup>r</sup> 97.57±0.86 95-100	<sup>r</sup> 97.57±0.86 95-100	<sup>r</sup> 97.57±0.86 95-100	<sup>r</sup> 97.57±0.86 95-100	
	Critical Difference(CD)	(5%) 6.067			(1%) 7.974		

Superscripts a,b,c,d,e indicates significant differences between concentrations (amongst the rows)  
Superscripts p,q,r indicates significant differences between different fungi (amongst the columns).

**Table 5:** Overall Effect Rate (OER)

S. No.	Fungus	OER (At 5×10 <sup>8</sup> conidia/litre)	OER (At 1×10 <sup>8</sup> conidia/litre)
11	MRO	100×(1-102.85/1578.57×11.8/96.8)= 99.20	100×(1-214.85/1578.57×40.6/96.8)= 94.29
22	MRN	100×(1-34.287/1578.57×38.60/96.8)=99.13	100×(1-50.714/1578.57×89.4/96.8)=70.35

*Metarhizium anisopliae*, a deuteromycete fungus, a general pathogen, that attacks diverse group of insects (Quesada-Moraga *et al.*, 2004). This fungus invades the host arthropod body through the cuticle and subsequently reproduce in it; thus favouring horizontal transmission and may also have an effect on the reproduction of the organisms being attacked (Quesada-Moraga *et al.*, 2004). Similarly *Metarhizium anisopliae* decreases the oviposition of the treated ticks and hatching could be interpreted as the vertical transmission in the future progeny of arthropods (Kaaya, 2000, Quesada-Moraga *et al.*, 2004). Samish *et al.* (2001) observed that high pathogenicity of *M. anisopliae* to various developmental stages of *R. sanguineus* and *R. appendiculatus*. Similarly they also reported that *M. anisopliae* isolates caused a subsequent secondary infection in subsequently emerging stages and can also prevent egg laying by engorged *R. sanguineus* tick. Through these observations author further opined that fungal infection may be transmitted from an infected to a non infected host, both among tick at the same stage of development and also among ticks of different stage of development, indicating that vertical and horizontal transmission of *M. anisopliae* fungus. Similar type effect (vertical and horizontal) has been observed in the present study on *R. (B) microplus* ticks. Combining together all the studies, it is proved beyond doubt that, *Metarhizium anisopliae* is having horizontal and vertical modes of transmission in the tick host. Thus *Metarhizium anisopliae* fungus appears to be effective in controlling tick population in the same generation as well as in the next generation. According to Broglio-Micheletti *et al.* (2012), *Metarhizium anisopliae* gives the result and causes the mortality in less time with low concentration.

Present study results proved that fungus tested is found to be the promising biological control agents against an important tick pest of cattle. Hence present research has two important components, one as tick and other as fungi tested as BCAs. The use of entomopathogenic fungi can be adapted both to environmental conditions in the region in which they will be used and to ticks, which served as a substrate for reproduction, may be useful in the implementation of *in vivo* biological control programmes against ticks (Fernández *et al.*, 2017). Potency and activity of the fungus is largely dependent on factors such as temperature and humidity. At a high temperature, low humidity fungus will not work.

In the present study *Metarhizium anisopliae* in 2 forms as old and new were evaluated. New means the fungal conidia which are recently harvested from the growing culture media and old means conidia harvested before 4 years from the growing culture media and stored in dark place away from the sunlight at room temperature. The idea behind testing new and old is to see

- a) Whether both old and new conidia has the similar potency
- b) To judge that for how much period conidia retains same potency after storage.

After study it was observed that, whether the fungus is new or old at par efficacy was recorded, though numerically slight differences were in evidence. Similarly after four years of storage, fungi hold the good potency. This species of entomopathogenic fungi established as BCA are having good amount of activity and can exert as cidal (killing) effect on adult ticks. Frazzon *et al.* (2000) developed the formula to scale the magnitude of effect of fungus on *B. microplus* ticks by calculating OER (Overall Effect Rate). By using the same formula the OER has been calculated.  $OER = 100 \times [1 - W_{Et}/W_{Ec} - W_{Lt}/W_{Lc}]$ , where  $W_{Et}$  is the egg laying capacity of infected ticks;  $W_{Ec}$  the egg laying capacity of non-infected ticks (control);  $W_{Lt}$  the egg fertility of infected ticks;  $W_{Lc}$  the egg fertility of non-infected ticks. From the data of the Table 5, it can be assessed that both fungal treatment showed significant activity against the *R. (B.) microplus* ticks ranging between 70.35-99.20% OER, which was remarkable and proving this fungi as potential biological control agents.

## Conclusion

The *Metarhizium anisopliae* fungus tested showed significant activity in terms of mortality of adult ticks, reducing egg laying capacity, less hatchability and less hatchability of treated eggs. The fungus when applied tick eggs caused the infection which resulted in reducing the hatchability of treated eggs and therefore if used at the sites where tick deposits the eggs, can prove as best biological control. The activity was optimum at 5g/l and hence it can be recommended for tick control programmes, subject to the evaluation under field conditions. When old stock of the fungus was evaluated, it had given at par activity with their respective newly harvested conidia, indicating that, if fungal powder is stored away

from the sunlight at room temperature in dry conditions, it can hold activity for more than 4 years. Thus present study concludes that *Metarhizium anisopliae* is better choice as ovicidal as compared to tickicidal in the tick control programmes. Efficacy of fungal treatments was found to be concentration dependent. Hence it is concluded that minimum concentration of  $5 \times 10^8$  conidia/litre can be recommended to achieve desired effect and can be incorporated in the integrated pest management approach.

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