Potentiality of Novel Strain of *Metarhizium anisopliae* (*Metarhizium anisopliae* Strain DULS TTRA) against *Odontotermes obesus*, A Pest of *Camellia sinensis* (L.) O. Kuntze

Sangeeta Hazarika¹, Dipsikha Bora¹, Bichitra Kumar Barthakur²

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**ABSTRACT**

*Odontotermes obesus* (Rambur) is an important termite pest of tea of North East India. The current study shows that a novel strain of *Metarhizium anisopliae* strain DULS TTRA, Accession no. KT 119358) a soil fungus is highly pathogenic to both worker and soldier caste of *Odontotermes obesus* in in-vitro condition. Efficacy of the experimental fungus further assessed by comparing with commercial formulation showed to have less LT50 and LC50 than the established virulent strain. We recommend the strain as a potential bio-control candidate against *Odontotermes obesus*.

**Key words:** *Metarhizium anisopliae* strain DULS TTRA, Mortality, Tea, Termite.

**INTRODUCTION**

Out of nine species belonging to six genera of termites reported from tea plantation areas of NE India, *Odontotermes obesus* (Rambur) is of frequent occurrence in tea plantation areas of both North Bank and Cachar Agro climatic tea growing zones and are responsible for considerable damage with significant effect on the productivity of the plant, leading to the loss of the capital of here. They generally confined to scattered bushes and normally clean out dead, dying woody tissues of the stem and bark of the bushes of the tea plant, *Camellia sinensis* (L.) O. Kuntze which leads to further death of adjoining healthy tissues. Continuous removal of such tissues leads to the formation of cavities and hollows in the stem and with the increasing damage, the cavities and hollows are enlarged and later filled up with earthen materials carried by the termites (Barua, 1989).

Tea industries mostly rely on chemical pesticides like cyclodienes, chlorpyrifos, deltamethrin, thiamethoxam, imidacloprid, endosulfan etc for management of termite populations (Choudhary et al., 2005). But in order to avoid the environmental and human health hazards caused by the chemical pesticides and particularly eliminate the pesticide residue in made tea, a search for safe, cheap and viable alternative termite management strategy in tea plantations has demanded serious concern. The use of entomopathogenic fungi is one such suitable avenue for termite management in tea plantations.

The soil environment is an important reservoir for a wide variety of pathogenic fungi, which play significant role in the management of agricultural and forest pests. Knowledge on the occurrence and distribution of indigenous pathogenic fungal species in a particular area is very essential as they can further use as biocontrol agent against different pests and pathogens of the same area. Moreover, local strains of particular area are observed to be more efficient than the commercial one isolated from other ecosystem (Babu and Kumhar, 2014). They also reduce the risk of significant impact on non target organism compared with exotic pathogenic isolates.

Based on our presumptions, the present work was undertaken with the objective to evaluate the pathogenicity and efficacy of an indigenous novel strain of *Metarhizium anisopliae* (*Metarhizium anisopliae* strain DULS TTRA, Accession no. KT 119358) isolated from termite non-infested tea soil against *Odontotermes obesus* in order to identify suitable entomopathogen to be used as bio control agent against termites.

**MATERIALS AND METHODS**

**Isolation and identification of fungal isolate**

Experimental fungus was isolated from the soil sample collected termite non infected areas of Naharani Tea Estate, Assam by following serial dilution plate method (Johnson and Curl, 1972) using Rose Bengal Chloramphenicol (RBC) agar medium. For confirmation of the identity of the fungus,
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culture of the fungus was sent to Xceliris Labs Ltd., Ahmedabad and identified as *Metarhizium anisopliae* 18S rRNA sequence and identified as *Metarhizium anisopliae* 18S rRNA sequence after DNA sequencing and Basic Local Alignment Search Tool (BLAST) with NCBI Gen Bank database and later its 18S rDNA sequences were submitted to NCBI GenBank. After validation of the sequences, the strain assigned as *Metarhizium anisopliae* strain DULS TTRA, with Accession no KP893281 by Gen Bank.

**Preparation of plate culture and stock conidial suspension**
Plate culture of the fungal isolate was done on Potato Dox Agar medium. 14 days old culture of the fungal isolate was selected as standard culture as experimentally observed to be sufficient time to mature and sporulate (Hazarika, 2015). Stock conidial suspension was prepared from above culture by scraping the fungal surface with a sterile surgical blade and then conidial clumps, mycelium were suspended in a solution of 3.0 mM KH₂PO₄ with 0.02% Tween 80 which later filtered through sterile nylon cloth to remove the mycelial debris and large clumps. Concentrations of the conidia of the stock conidial suspension was further determined using a Neubauer Haemocytometer under Phase Contrast Microscope.

**Collection and maintenance of *Odontotermes obesus* under laboratory condition**
The experimental termite samples of *Odontotermes obesus* were collected from the tea cultivation areas located inside the campus of Dibrugarh University, Assam, India in plastic containers along with their nest materials. After the collection they were transferred to aluminium trays. Moist termite infested wooden sticks were given as food and moist cotton was placed in the trays to maintain humidity. The trays were covered with fine mesh cloth that allows air ventilation but prevents the escape of the termites and placed in a dark cool area for conducting the experiments. Periodically, dry wooden materials were also provided as fresh stock of food.

**Pathogenicity trail of experimental fungus**
The pathogenicity trial was performed according to Singha *et al.* (2011). The experimental fungal isolate was cultured on Potato Dox Agar (PDA) medium for 14 days. Three replicates, each with ten termites of worker and soldier castes of similar size individually were carefully selected from rearing trays and placed separately on culture petri plates containing the experimental fungal isolate. After 15 minutes of exposure, each group of the termites was carefully transferred separately from the culture plates onto a second sterile petri plates (9 cm) lined with moist Whatman filter paper and incubated at (25±2)°C. Mortality of the experimental soldier and worker caste termites were recorded separately at an interval of 24 hrs up to the fifth day after exposure. The dead termites from each petri dish were removed everyday during the experimental period and kept in separate petri dishes lined with moist filter paper by using sterilized forceps. The dead termites were kept at (25±2)°C for development of overgrowth of the fungus on the cadavers. Pathogenicity of the fungus was further confirmed by Koch’s postulation.

**Assessment of lethal dose**
The fungal isolate was applied against on both soldier and worker caste of *Odontotermes obesus* separately under laboratory condition. For determination of LC50, a stock conidial suspension of the fungus was prepared from 14 day old PDA culture and diluted six times by serial dilution technique. 0.5ml of different concentrations of the conidial suspensions of the test fungus were transferred separately to sterilized petri plates lined with moist filter paper, containing the experimental termites and maintained at (25±2)°C in the dark. The resulting mortality of termites was recorded at an interval of 24 hrs till the 5th day. The dead termites were immediately removed from the petri plates to prevent contamination. Tericon-M.A. was used as recommended control and sterile water was used in control. All the work was conducted in front of laminar air flow system.

**Assessment of lethal time**
Three equivalents conidial concentrations (1x10⁷, 1x10⁸ and1x10⁷ conidia/ml) of the test fungus prepared from the stock conidial suspension were used in this study. Time required for 50% mortality of both worker and soldier caste of *Odontotermes obesus* in each concentration in treated filter paper was determined. Two groups of control (Tericon-M.A. and sterile water) were maintained during the study. Mortality of termites was recorded at an interval of 24 hrs till 5th day of exposure.

**Statistical analysis**
The Abbott’s formula (Abbott, 1925) was applied to correct the percent mortality in the control. Software SPSS 13.0 was used for the statistical data processing. Probit analysis was carried out to estimate LT50 and LC50.

**RESULTS AND DISCUSSION**
The pathogenicity test revealed that experimental novel strain of *Metarhizium anisopliae* (*Metarhizium anisopliae* strain DULS TTRA, Accession no. KT 119358) is highly pathogenic and virulent to both castes of *Odontotermes obesus* causing hundred percent mortality by 3rd and 5th day respectively in worker and soldier castes of *Odontotermes obesus* under in vitro condition (Fig 1 and 2).

The relative virulence of the identified entomopathogenic fungi of 14 days old pure culture on worker and soldier castes of *Odontotermes obesus* treated with spore suspension by filter paper method were measured in terms of median lethal concentration fifty (LC50) presented in Table 1. LC50 of *M. anisopliae* strain DULS TTRA against both the worker and soldier was lower than the Tericon M.A.

Differences also observed in fungus-induced mortality to termites with three equivalent conidial doses. The results revealed that mortality (%) of both castes of termite at
different experimental concentrations of test fungi were significantly different from each other (p<0.001) after 5th day exposure (Table 2). However, in response to contact with Metarhizium anisopliae strain DULS TTRA in petri dishes, Odontotermes obesus exhibited significant higher mortality of 100% in both worker and soldier caste compared to Tericon M.A. (84% in worker and 82.14% in soldier) at higher concentration of 1x10^9 conidia/ml. The mortality percentage, at the concentration of 1 x 10^8 and 1 x 10^7 conidia/ml of the experimental fungi were also in higher in comparison to that of the recommended control at the same concentration in vitro condition (Table 2).

The median lethal time (LT 50) i.e., the time taken for the death of 50% of worker and soldier caste of Odontotermes obesus in response to the action of the experimental fungi at different concentrations of 1x10^7 to 1x10^2 conidia/ml are presented in Table 2. The results revealed that the LT50 values of Metarhizium anisopliae strain DULS TTRA was significantly lower than that of the recommended control at all the conidial concentrations when applied against both worker and soldier castes.

Potential use of M. anisopliae as biocontrol agent against termite has been a challenge (Chouvec et al., 2011) and identification of new virulent fungal strain, decision making as regard the manner of use of the isolate and termite grooming behaviour are certain important issues that require concern. The fungal virulence is reported to have a strong correlation with the fungal removal by grooming behaviour (Yanagawa et al., 2010) and hence the challenge posed by termite’s efficiency to avoid fungal infection rendering them fungal resistant, is possible to be averted by finding out of the highly virulent new strain. The pathogenicity study under in vitro condition indicated Metarhizium anisopliae strain DULS TTRA (Accession no KT 119358) is virulent against both soldier and worker caste of Odontotermes obesus. The study revealed that isolated M. anisopliae strain DULS TTRA caused 100% mortality of worker and soldier caste of Odontotermes obesus by the 3rd and 5th day respectively (Fig 1 and 2). Earlier, Singh et al (2011) tried to control the tea termite Microtermes obesi predominantly present in Barak valley, Assam by using recommended biocontrol agents namely Metarhizium anisopliae (IARI) and Metarhizium anisopliae (PDBC). They reported that both strain caused 100% mortality of the worker caste of termite by the 8th day only of pathogenicity.

For a fungal pathogen to be successful, the fungus has to establish itself before killing the insect. However once established, saprophytic fungi may again grow and develop on the cadaver. In case of M. anisopliae var anisopliae, the toxin level produced in vitro is reported to influence the killing in selected insects (Kershaw et al., 1999). The mortality caused by the indigenous fungal strain in our study, might be due to secretion of destuxin and observed enhanced mortality after the second day of exposure (Fig 1 and 2) might be due to rapid penetration of the cyclic peptide toxin, destuxin into the termite’s hemocoel (Krutmuanga and Mekechay, 2005). After four to five days of infection, white mycelia of M. anisopliae strain DULS TTRA developed and after six to seven days, green conidia appeared around the insect cadavers (Plate 1).

Susceptibility of termites to fungal infection is dose dependent (Rosengaard and Tranillo, 1997). However, the mortality occurring due to entomopathogenic fungi depends not only on the concentration of the conidial suspension but...
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Table 2: Mean mortality (\%) and LT$_{50}$ of three equivalent conidial concentrations (conidia/ml) of \textit{Metarhizium anisopliae} strain DULS TTRA and Tericon M.A. against \textit{Odontotermes obesus} under \textit{in vitro} condition.

| Fungal species | Treatments | Mortality (\%) after 5th day | LT$_{50}$ (hour) |
|----------------|------------|-----------------------------|-----------------|
|                | Worker     | Soldier                     | Worker          | Soldier         |
| \textit{Metarhizium anisopliae} strain DULS TTRA | 1x10$^7$ | 100.00 ± 0.00 | 26.60 | 28.72 |
|                | 1x10$^8$ | 96.42 ± 0.57 | 30.66 | 34.80 |
|                | 1x10$^9$ | 92.85 ± 0.57 | 33.74 | 40.23 |
| \textit{Tericon-M.A.} | 1x10$^9$ | 84.00 ± 1.15 | 34.49 | 49.18 |
|                | 1x10$^8$ | 80.00 ± 1.52 | 38.11 | 55.84 |
|                | 1x10$^7$ | 76.00 ± 0.66 | 42.01 | 59.02 |
| \textit{Water} | -          | 16.66 ± 0.57 | 6.66 ± 0.57 | - |

Within a column, means followed by different letter are significantly different (P < 0.001).

Also on the time and manner of exposure (Ansari et al., 2004; Ahmed et al., 2009). In our study, \textit{Metarhizium anisopliae} strain DULS TTRA caused 100% mortality of worker caste at the minimum concentration of 1x10$^7$ conidia ml$^{-1}$ by the 5th day (Table 2). As mycelia development also took place by 4th to 5th day, therefore the strain could successfully establish before the death of the termites. In contrast, Dong et al. (2009) isolated a new virulent \textit{Metarhizium anisopliae} variety \textit{anisopliae} from \textit{Odontotermes formosanus} in China and reported it to cause 100% mortality by the third day post-inoculation at a much higher concentration of 3x10$^8$ conidia/ml against the subterranean termite, \textit{Odontotermes formosanus}. Pik-Kheng et al. (2009) however reported pathogenicity of isolates of \textit{M. anisopliae} causing 100% mortality at 1x10$^7$ conidia ml$^{-1}$ within 3 days post-inoculation but against on a different genus, \textit{Coptotermes curvignathus}. Sileshi et al. (2013) reported that at the highest concentration of 1x10$^9$ conidial ml$^{-1}$, both \textit{B. bassiana} and \textit{M. anisopliae} isolates caused 100% mortality of \textit{Macrotermes} worker termite population after sixth days of exposure.

**CONCLUSION**

Based on the results and discussion above, we are reporting a novel pathogenic strain \textit{M. anisopliae} strain DULS TTRA (Accession no. KT 119358) isolated from the tea garden soil of Assam to be highly effective against \textit{Odontotermes obesus}, a pest of \textit{Camellia sinensis} (L.) O. Kuntze. However, further study on mass production and testing of germination, growth on various domestic and agricultural wastes, self life study and field efficacy of the experimental fungus is required for commercial utilization of this potential biocontrol agent in management of termite population in tea plantation areas of Assam.

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