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Scanning Electron Microscopy (SEM) Study Showed the Mode Action of *Metarhizium anisopliae* on *Odontotermes obesus*

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The present study was done to investigate the histopathological effect of the entomopathogenic fungi, *Metarhizium anisopliae* on *Odontotermes obesus* using Scanning Electron Microscopy (SEM). Adult worker termites were inoculated with 1×10^9 conidia/ml suspension of the fungi and maintained at $25 \pm 1^\circ\text{C}$ temperature and 80 % relative humidity. SEM study showed that when this mitotic (asexual) spore (called conidia) of the fungus came into contact with the body of the termite, they germinated and the hyphae of the fungus that emerged penetrated the cuticle. The hyphal penetration of the fungi through the insect cuticle as well as proliferation formed a dense network of mycelium in the insect cuticle. This fungus also invaded the host through the integument and caused death of the insect. This lethal effect to the insect is very likely to be aided by the production of insecticidal cyclic peptides (destruxins) by the fungus. SEM study revealed that it is a convenient tool to observe the mode of action of entomopathogenic fungi and to observe how they are able to colonize and infect the host.

Introduction

Termites are one of the most important pest of agricultural, horticultural, plantation crops, forest trees etc. Conventional termite control depends largely on the use of chemical insecticides. However many of these insecticides are banned now a days because of its ill effects to non-target insects as well as their persistence in the environment.

Due to increasing cost of the pesticides, outbreak of secondary pests, insecticidal resistance and pollution problems search for alternatives to chemical pest control has assumed greater importance in recent years.

One such alternative method is biological control using entomopathogenic fungi (Khan *et al.*, 1993). Termites are considered as good candidate for control with the entomopathogenic organisms because they live in a conducive environment, humid, minimal diurnal temperature, fluctuations, crowded and with considerable social interaction (Delante *et al.*, 1995; Creffield, 1996). Biological control of termites using entomopathogenic fungi have been reported by Lai *et al.*, 1982; Hanel and Watson, 1983; Gurusubramanian, *et al.*, 1999; Rath, 2000.

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to

generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition and crystalline structure and orientation of materials making up the sample. The objective of the present investigation was to study the external development of *Metarhizium anisopliae* treated on the termite body using SEM.

Materials and Methods

Rearing of termites

Adult workers of *Odontotermes obesus* (Termites) were collected from the Experimental Garden for Plantation Crops (EGPC), Assam Agricultural University, Jorhat. Collected termites were reared at room temperature in plastic containers with sand and wetted corrugated cardboard until use.

Source of fungal isolate

The pure culture of the fungus, *Metarhizium anisopliae* was obtained from the insect isolates by Pegu *et al.*, (2012). These fungi were then sub cultured in Potato Dextrose Agar (PDA) media and incubated at $25 \pm 1^\circ\text{C}$ for 5-7 days. Twenty (20) ml of the sterilized PDA media was poured aseptically in sterilized petridishes. On solidification of the media the petridishes were inoculated with a loop full of fungal mass from the pure culture and then incubated at $25 \pm 1^\circ\text{C}$ for sporulation. Isolated and purified cultures were maintained by periodical transferring in fresh PDA slants.

Fungal suspensions

Fungal propagules were scrapped out from the surface of the mass culture with the help of a fine brush. A stock suspension was prepared by placing harvested propagules in pure

distilled water. Homogenous suspension of viable conidia was filtered through double layered muslin cloth to remove the mycelial mat and suspended in 0.05 per cent aqueous Tween 80. A Neubeur Haemocytometer was used to estimate the conidial concentration and subsequent appropriate dilutions were made thereof.

Bioassay

Twenty numbers of termites were transferred to petridishes lined with filter paper. Termites were inoculated with a suspension 1×10^6 conidia/ml of *M. anisopliae* and maintained at $25 \pm 1^\circ\text{C}$ and 80% relative humidity. The termite mortality was observed at 24 h intervals for up to 7 days. Dead termites were incubated in a humid chamber to confirm growth of the fungus on their cadavers. A batch of uninfected termites was maintained as a control.

Preparation of treated termite samples for scanning electron microscopic examination

The infected death termites were held in absolute ethanol for one month. It was checked periodically so that the alcohol does not evaporate from the container.

The insect was then removed, washed gently with absolute ethanol and allowed to dry in a covered dish (to avoid dust) for at least one week.

This step allows the alcohol that has replaced the insects' blood to evaporate. Then, the insect was placed on a mount on a spot of carbon paint to hold it in place and coated with a layer of gold. Samples were observed under SEM (JEOL, JSM- 6390LV) and photographs were taken to get detailed morphological changes of the insect body at Department of Physics, Tezpur University, Assam.

Results and Discussion

At low magnification the infected termite showed distortion and deformation of the whole body having no proper orientation. Distortion was more in abdominal part of the insect body. There was shrinkage in the head cuticle and deformity of head sensilla (Fig. 1b-c). At higher magnification the fungal colonies were clearly visible throughout the body parts (Fig. 1d). The spores were characterized by green colour. The fungal hyphae together formed a dense network of mycelium. The spores and hyphae of the fungus were also seen in the body cavity.

The formation of haloes around fungal conidia and germination tubes on the insect cuticle was also observed with conidia adhesion and germination. A thickening of the extremity of the germ-tube, characterizing the formation of aspersoria of the fungus was also observed during penetration by the fungus. The mycelial extrusion of *M. anisopliae* was more in the intersegmental areas resulting in a process of cuticle degradation along the whole body of the insect. The whole body was covered by *M. anisopliae* conidia.

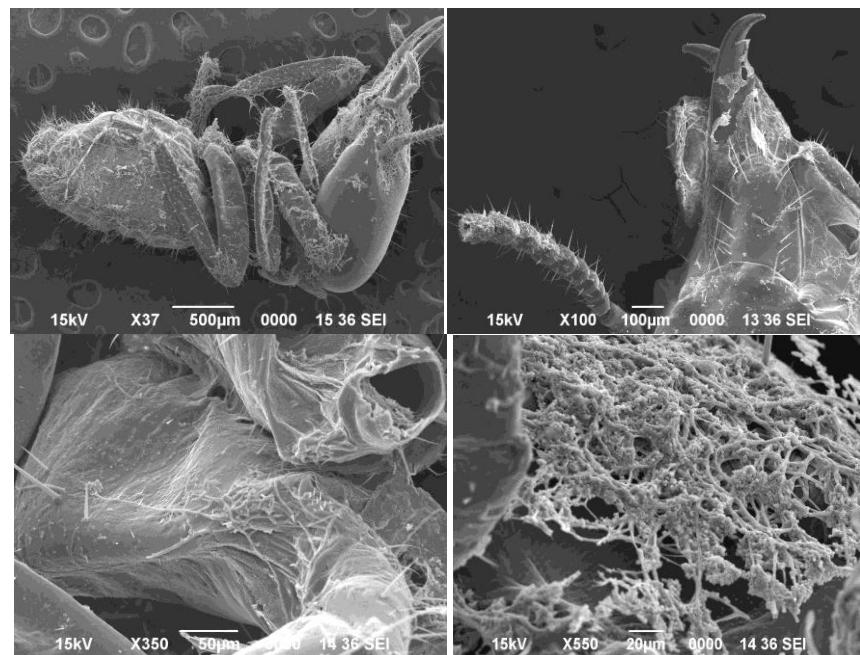
Scanning electron microscopy (SEM) of termite treated with the fungus, *M. anisopliae* clearly revealed adhesion and penetration structures in the infected termite. Infection of termite by the fungus, *M. anisopliae* requires adhesion, penetration into the host (St Leger, 1993) and establishment of the pathogen in the host (Charnley, 1989; Sampson *et al.*, 1988). Penetration through the host cuticle is the mode of entry for most entomopathogenic fungi (Charnley, 1984). During fungal infection, the first step prior to penetration is the adhesion of fungi to the host cuticle (Boucias and Pendland, 1991). Fragues (1984) suggested adhesion to occur at three successive stages: adsorption of the fungi propagules to the cuticular surface, adhesion

of the interface between propagules and epicuticle and fungi germination and development at the insect cuticular surface, until appresoria are developed to start the penetration stage. *M. anisopliae* produces some cyclic peptide toxins, destruxins, which exhibit a variety of insecticidal actions by Sharif *et al.*, (2010) and thirty-eight destruxin analogues have been reported to date (Schrink and Vainstein, 2010).

The formation of haloes on the insect cuticle at the germination and penetration points was also observed. The occurrence of these haloes may be due to production and excretion of exoenzymes by the entomopathogen during the infective process. The combination of proteolitic enzymes and chitinase produced by the fungal mycelium may digest the insect cuticle, facilitating the penetration of the insect integument. Once the fungus penetrates the host; it produces toxins that overcome the insect immune system.

Appressoria of *M. anisopliae* was also formed in the body of the infected termite. This was characterized by a thickening of the extremity of the germ-tubes. This Thickening is probably due to the translocation of the conidial cytoplasmatic content to facilitate the enzymatic synthesis necessary for the penetration phase. In some areas such as the insect head, extensive growth of germ-tubes was observed, probably due to resistance to fungal penetration in these areas with more heavily sclerotized cuticle. The formation of haloes on the insect cuticle at the germination and penetration points was also observed. An enzyme of the chymoelastase group (Pr1) has been implicated in cuticle degradation (St. Leger *et al.*, 1988). According to these authors, *M. anisopliae* uses up to 78% of the protein synthesis for the production of this important enzyme in the process of penetration. Pr1 is a key factor in the virulence of this fungus.

Fig.1 SEM image of *Metarhizium anisopliae* infected termite (a-d)



a. *M. anisopliae* infected adult termite, b-c. Shrinkage and deformity in the cuticle and body parts, d. Growth of fungal structure arising from infected termite body

Despite the standardization in fungal application, the inoculums that reach the insects are variable. This variation may interfere with the extent of colonization of the insect, the speed of mycelium growth, and conidiogenesis, resulting in differences in the mortality of the insects.

The rates of conidia germination and penetration of the insect integument have been shown to be related to the virulence of the fungi and the susceptibility of the insect host (Pekrul & Grula, 1979; Fargues, 1984). Fungal infection by *M. anisopliae* on the termite body begins when conidia (asexual spores) attach to insect's cuticle.

The spores germinate and penetrate the insect's skin and enter the host. Once the fungus penetrates the host, it produces toxins that overcome the insect immune system. Thereafter, the hyphae penetrate through the cuticle to the outside and cause green sporulation on the insect's body.

M. anisopliae has a high potential for exploitation into a commercial bio insecticide in India. Their low toxicity to humans compared with chemical pesticides is an advantage, especially in India, where the risk of misuse of pesticides is very high. Further studies should include optimisation of dosage for field application and treatment of large area in different agro ecological zones, and socioeconomics. In conclusion, the results obtained in these experiments establish the pathogenicity of entomopathogenic fungi *M. anisopliae* on termite as biological control agent. Biological control with pathogenic fungi is promising alternative to chemical control against the termites.

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