

## PATHOGENICITY AND DEVELOPMENT OF *ASPERGILLUS FLAVUS* IN VARIOUS SPECIES OF TERMITES

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**Abstract:** A locally isolated strain of *Aspergillus flavus* was found highly pathogenic to *Microcerotermes championi*, *Bifiditermes beesoni* and *Heterotermes indicola*. Pathogenicity was first tested against the workers of *M. championi* by overhead culture method (exposure) and surface culture method (crawling). As there was no significant difference in the mortality rate of termites duly exposed to fungal culture for 10, 20 or 30 minutes; further experiments were carried out using only crawling method for the nymphs of *B. beesoni* and workers of *H. indicola*. Higher mortality of termites occurred when they crawled for a longer period. Histopathological studies showed that their mycelia branched into body cavity and attacked all the organs including the ganglion of nerve cord, when entry mode was through integuments. However, when the conidia entered through oral route, they were seen tapped by cytoplasmic masses and strands developing in the gizzard and gut of the termites were observable. The comparative data of  $LT_{50}$ ,  $LT_{90}$  and slope of regression line of various species of termites showed that *M. championi* was most susceptible to *A. flavus* than *H. indicola* and *B. beesoni*.

**Keywords:** *M. championi*, *B. beesoni*, *H. indicola*, biological control, fungus, histopathological studies.

### INTRODUCTION

Termites as pests, are well known for the damage they cause to agricultural crops, stored products, forests and wood work of buildings. Many efforts have been made to control the termites by chemicals. The chemicals destroy termites in a specific area and are unable to destroy the source of termites. According to Vypijach *et al.* (1972), the

microorganisms possess specificity of action on insects and are harmless for useful animals, plants and men. There are at least six species of fungi, infecting more than 200 insect species in seven insect orders (Zimmermann, 1993). Several ectoparasitic genera, *Ectomyces*, *Termiteria* (Tate, 1927, 1928) and *Antennopsis* (Buchli, 1951) are recorded to cause a mycosis in termites. Krejzova (1976) isolated a strain of *Paecilomyces fumoso-roseus* from *Zootermes formosanus* and *Reticulitermes lucifugus*. A few parasitic fungi on termites were also reported and described by several investigators (Bao and Yendol, 1971; Kimbrough *et al.*, 1972; Yendol and Rosario, 1972; Rossi, 1974; Rossi and Rossi, 1977a, b; Blackwell and Kimbrough, 1976). Although the mechanism of disease resistance in termites has been reported in the form of inhibition of fungal spores (Rosengaus *et al.*, 2000); the pathogenicity of the fungi to termites is widely accepted. The causal agent of green muscardine diseases, *Metarhizium anisopliae* is considered as naturally occurring pathogen for the control for termites (Hanel and Watson, 1983). Similarly Rosengaus and Traniello (1997) also reported the pathobiology and disease transmission on damp wood termite infected by *M. anisopliae*.

According to the Lenz (1968) a strain of *Aspergillus niger* was known to produce at least two toxins and one of them was found to be toxic to the termite *H. indicola*. Khan *et al.* (1994) also reported the pathogenicity of *A. niger* against various species of termites. In laboratory test Beal and Kais (1962) experimentally infected two subterranean species *Reticulitermes virginicus* (Banks) and *R. flavipes* (Kollar) with *A. flavus*. Sannasi (1968) observed dead queen and king of *Odontotermes obesus* and found *A. Flavus* as the causative agent. In the present studies too *A. flavus* is reported as a potential pathogen of termites and the pathogenicity is confirmed through histological studies of various infected tissues of different species of termites.

## MATERIALS AND METHODS

In order to check the pathogenicity of the unknown isolated microorganisms, according to Bucher (1973) they may first be tested on the same species from which they were originally obtained. A similar pattern was followed in the present study.

### ***Collection of Samples***

During the course of study, many samples of various species of termites (sick or dead) were collected from the field and kept in sterile Killer jars (2 lbs capacity) containing moist filter papers. The samples were then brought to laboratory for further observations.

### ***Isolation of Pathogens***

The termites were surface sterilized by 5.25% sodium hypochlorite as described by Bao and Yendol (1971). The infected, sick or dead termites were crushed in sterile distilled water and their suspensions were prepared. An inoculum of the suspension was directly streaked on the agar media. Dilutions of suspensions were prepared and a pour plate method was used as mentioned by Cruickshank *et al.*, (1975). In addition to nutrient agar, Sabouraud's dextrose and Czapeck's agar media were also used for observing growth of fungi. Pure cultures of fungi were maintained on Sabouraud's dextrose (SD) and yeast agar (YA) medium as described by Yendol and Rosario (1972). The media used were incubated at 25-30 °C up till 72 hours. Different colonies growing on the medium were isolated and inoculated on the slants to preserve a pure culture. Inocula of isolated pure cultures were tested separately for their pathogenicity against various species of termites.

### ***Identification of Fungi***

Suspensions of dead or infected samples of termites were observed under microscopes. Their smears were also prepared. The isolated pure cultures were stained and examined for their morphological characters. For slide culture of fungi, blocks (25mm<sup>2</sup>) of a medium (SD & YA) were used as described by Riddell (1951). The fungi were stained with Lactophenol blue solution. The sprouting culture of fungi was teased on the slide with a drop of 95% ethyl alcohol and then the stain was applied. The slides were fixed over the flame for few seconds. After keeping it overnight, the excessive stain was blotted from the edges of cover slips. In order to check the growth and manner of development of fungi on slides, daily observations were taken by microscope. The identification of fungus was made with the help of a key mentioned by Raper and Fennell (1965). *A. flavus* was isolated from a sample of *B. beesoni* collected from the stem of *Pyrus communis*, growing in an orchard near Lahore. Its pathogenicity was tested against *M. championi*, *H. indicola* and *B. Beesoni*.

The fungus was grown on Sabouraud's dextrose and yeast agar medium for seven days. The inoculation of the fungus was carried out as described by Yendol and Rosario (1972). A quantity of 5ml. of sterile distilled water was poured in the culture and the conidial culture was suspended in the water. One ml of aliquot was taken and poured on SD and YA medium and incubated at 30 °C for 72 hours.

Pathogenicity of *A. flavus* was first tested against workers of *M. championi* by two methods. The overhead culture method (exposure) consisted of inverting the sporulating culture over termite and allowing conidia to fall upon the termites for a period of 10, 20 and 30 minutes. In the surface culture method (crawling), termites were placed directly on sporulating cultures and allowed to crawl over the fungal surface for 10, 20 and 30 minutes.

In each method, control groups were treated in the same fashion except that the fungal inoculation was omitted. When the inoculating period terminated, termites were transferred to other Petri dishes containing a double layered filter paper as a bed, slightly dampened with sterile distilled water.

#### ***Treatment of Data***

The response of *A. flavus* was recorded and results were plotted as regression lines, which were computerized by probit analysis. LT<sub>50</sub> and LT<sub>90</sub> were determined for comparative study while the slopes of regression lines (b) were determined in order to confirm the pathogenesis. Where the control mortality was between 5 to 12 percent, the percentage mortality was corrected by Abbott's formula as described by WHO (1975).

Probit analysis was then used to obtain LT<sub>50</sub>, LT<sub>90</sub> and regression equations. Details of the method of probit analysis been mentioned elsewhere (Khan *et al.*, 1992). Results obtained, where control mortalities exceed 12%, were discarded.

#### ***Microbiological Diagnosis***

In order to prove the microbial pathogenicity, Robert Koch's postulates were followed as described by Bucher (1973). The termites which died as a result of fungal infection were crushed and their smears were stained lactophenol blue solution. In order to observe the mode of action of the pathogens in the termites, histopathology was carried out as described by Vago and Amargier (1963). The histological sections of

various species of termites infected by fungi were stained using the technique of Hotchkiss (1948).

## RESULTS

The fungus, isolated from a sample of *B. beesoni*, had septate, colourless and branching hyphae. When the fungus was cultured on Sabouraud's dextrose agar medium, the conidial heads were light yellow-green or deep yellow-green to olive-brown. The conidial heads remained yellow-green and did not shift to brown or olive-brown color when the fungus was cultured on Czapeck's agar medium. Conidia were echinulate and appeared in chain form on sterigmata.

The culture slides of the fungus were prepared and observed daily. A conidial culture was stained by a drop of 95% ethyl alcohol and lectophenol blue stain. The characteristic feature of the fungi was studied in accordance with the key as described by Raper and Fennell (1965) and it was identified as *Aspergillus flavus*. Its pathogenicity was tested against workers of *M. championi*, *H. indicola* and nymphs of *B. beesoni*. Each test was replicated thrice. Histopathological studies were also carried out to see the mode of fungal attack.

### ***Tests against Microcerotermes championi***

*Microcerotermes championi* workers were divided into seven groups. Group 1 was kept as control. While groups 2, 3 and 4 were exposed to 72 hours conidial culture of the fungus for 10, 20 and 30 minutes, respectively. Termites of groups 5, 6 and 7 were allowed to crawl on conidial culture of the fungus for 10, 20 and 30 minutes, respectively.

At 24 hours following infection there appeared about 8%, 25% and 18% mortalities of termites caused by 10, 20 and 30 minutes of exposure to *A. flavus* conidial culture, respectively. There were about 15%, 18% and 48% deaths of termites at 24 hours following 10, 20 and 30 minutes of crawling of termites on *A. flavus* culture, respectively (Table I). The regression curves related to the mortality of *M. championi* caused by *A. flavus* are presented in Fig. 1 and the value of slopes was calculated in a range of 3 to 6.7. There was 50% deaths of termites at about 71 hours, 52 hours and 58 hours and 90% death occurred at about 145 hours, 138 hours and 125 hours following 10, 20 and 30 minutes of 'exposure' of termites to *A. flavus*, respectively. However, when termites were allowed to crawl on

*A. flavus* culture for 10, 20 and 30 minutes, the  $LT_{50}$  was calculated as about 55 hours, 56 hours and 24 hours and  $LT_{90}$  was 134 hours, 133 hours and 38 hours, respectively. These results indicated that *A. flavus* was pathogenic to *M. championi*. It was also noted that when *M. championi* workers were allowed to crawl for a longer period of time i.e., up to 30 minutes, their 50% and 90% mortalities occurred in a shorter duration of time (Table II). But there was no significant difference in the mortality rates of termites, when they were exposed to *A. flavus* culture for 10, 20 and 30 minutes. So further experiments were carried out using only crawling method.

**Table I: Mortality percentage of *M. championi* infected by 72 hours old cultures of *A. flavus***

Hrs. after infection	Gr. 1 Control	Gr. 2 Gr. 3 Gr. 4 Exposure for (min.)			Gr. 5 Gr. 6 Gr. 7 Crawling for (min.)		
		10	20	30	10	20	30
		24	5.33	8.23	25.16	18.14	15.36
48	6.67	23.71	32.06	37.85	37.74	27.81	97.33
72	6.67	39.19	60.37	46.32	59.01	63.24	100.00
96	6.67	51.88	71.71	70.37	71.71	73.15	
120	6.67	94.42	90.32	100.00	100.00	100.00	
144	6.67	100.0	100.00				

**Table II:  $LT_{50}/LT_{90}$  and value of slopes of Regression lines of *M. championi* Infected by 72 hours old culture of *A. flavus***

Inoculation period (minutes)	Exposure (overhead culture method)		Crawling (surface culture) method	
	$LT_{50}/LT_{90}$	Slope (b)	$LT_{50}/LT_{90}$	Slope (b)
10	70.6/145.2	$4.1 \pm 2.5$	55.4/133.8	$3.3 \pm 1.0$
20	52.5/138.2	$3.0 \pm 0.9$	55.9/132.8	$3.4 \pm 1.0$
30	57.8/125.2	$3.0 \pm 1.0$	24.5/38.0	$6.7 \pm 3.0$

Histopathological studies were carried out in order to see the mode of action and development of *A. flavus* in *M. championi*. *Aspergillus flavus* was originally isolated from dead nymphs and soldiers of *Bifiditermes beesoni*. The workers of *M. championi* were allowed to crawl on 72 hours old conidial culture of *A. flavus* for 20 and 30 minutes. A large number of

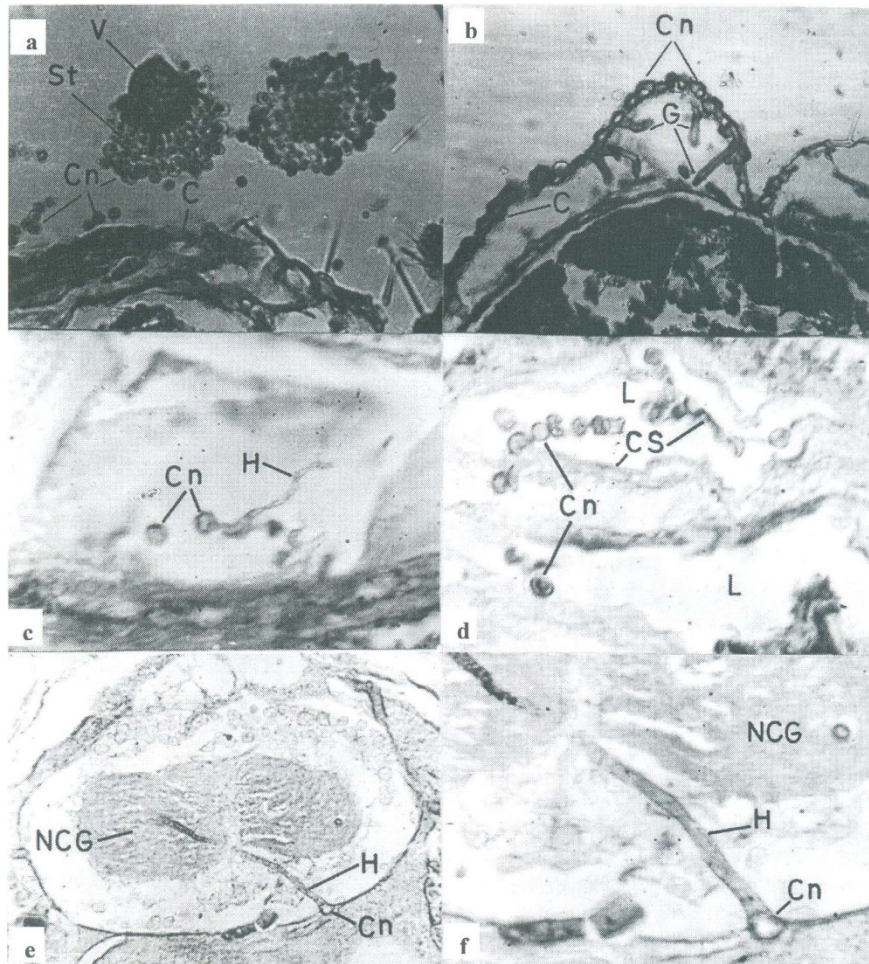
conidia were seen falling from sterigmata towards the cuticle of termites, where they aggregated up to 48 hours following infection (Plate-1, Fig. a). The conidia started germination and their germ tubes pierced through the cuticle at 72 hours following infection (Plate 1, Fig. b).

At 120 hours following infection, the hyphae developed in the body cavity and a few conidia germinated on the sheath of the ganglion of the nerve cord and their hyphae attacked the ganglion (Plate 1, Fig. e-f).

The conidia of *A. flavus* entered the alimentary canal of *M. championi* through food. They started germination in the lumen of the midgut at 72 hours following infection (Plate 1, Fig. c). Cytoplasmic strands were seen in the lumen of the midgut. Conidia were seen trapped in the cytoplasmic masses which developed at 120 hours following infection (Plate 1, Fig. d). It appears that the development of the cytoplasmic strands was a defense phenomenon in the host.

#### **Test against *Bifiditermes beesoni***

The nymphs of *B. beesoni* when allowed to crawl on *A. flavus* culture of 10, 20 and 30 minutes showed 12%, 17% and 28% mortalities at 24 hours following infection, respectively (Table III). The regression curves related to the mortality of *B. beesoni* are presented in Fig. 3. The value of slopes was calculated as almost equal (3.3) when the termites were allowed to crawl on *A. flavus* for 10, 20 and 30 minutes. The calculated values of LT<sub>50</sub> were about 77 hrs, 74 hrs and 55 hrs and their LT<sub>90</sub> were as 187 hours, 174 hours and 133 hours following 10, 20 and 30 minutes crawling of termites on *A. flavus* culture, respectively (Table-V). These results showed that *A. flavus* was fairly pathogenic to *B. beesoni* nymphs. These observations also showed that when the termites crawled for a longer period on fungal cultures, their mortality occurred in a shorter period of time. Histopathological studies were carried out to see the modes of actions of *A. flavus* against *B. beesoni*.



**Plate-1 Fig. a-f, *Aspergillus flavus* a, vesicle bearing sterigmata (after detaching from sterigmata, the conidia are falling and aggregating on the culture of *Microcerotermes championi*, x 400. b, conidia attacking cuticle and developing germ-tubes, x 1000. c, conidia germinating near the culture wall of gizzard, x 1000. e, conidia germinating the hyphae attacking the interior of nerve cord ganglion, x 4000. f, same as above, x1000. C, cuticle; Cn, conidia; CS, Cytoplasmic strands; G, germ tubes; H, hyphae; L, lumen; NCG, nerve cord ganglion; St, sterigmata; V, vesicle.**

These studies showed that after passing through the cuticle the hyphae of *A. flavus* branched into the body cavity of *B. beesoni* at 72 hours following infection (Plate 2, Fig. a). Several conidia were seen surrounded



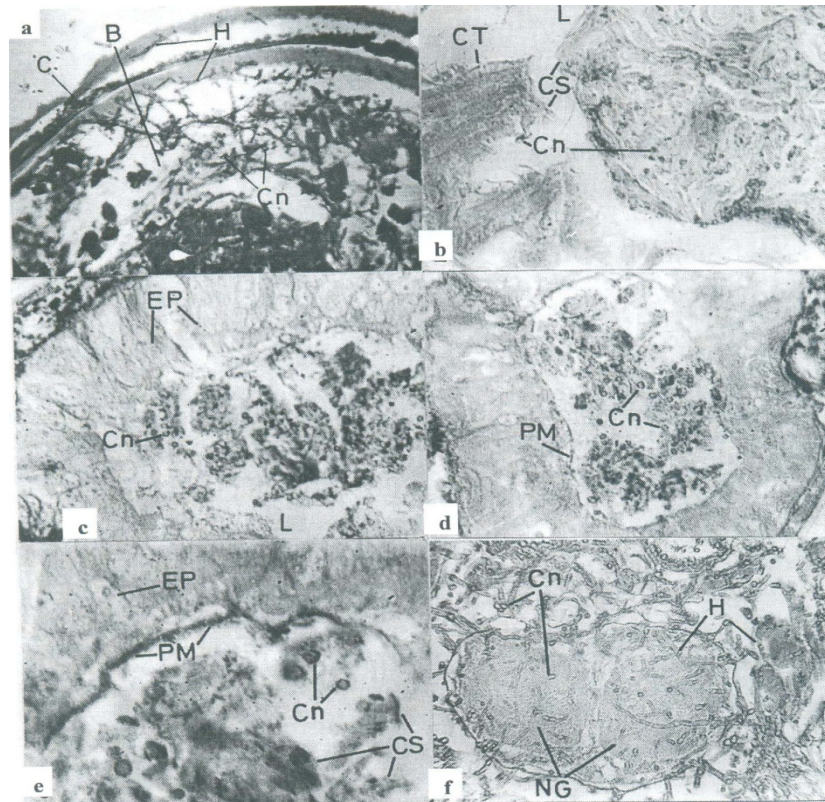
by cytoplasmic strands in the lumen of the gizzard (Plate 2, Fig. b). A conidium was also seen near cuticular lining of the gizzard.

At 120 hours following infection, in some sections, a large number of conidia were seen not only in the lumen of the midgut but also concentrated near epithelial cells (Plate 2, Fig. c.). In a few sections, a thick peritrophic membrane was seen around the epithelium of the midgut. Several conidia were seen trapped in a cytoplasmic mass or strands developing in the lumen of the gut (Plate 2, Fig. d and e). Moreover, a large number of conidia appeared germinating and their hyphae attacked the ganglion of nerve cord (Plate 2, Fig. f). The histopathological studies showed that when *A. flavus* attacked through the integument, *B. beesoni* was unable to offer any resistance. This was indicated by the fact that hyphae attacked all the tissues of the insect. However, when the conidia were ingested through food, the alimentary canal of the insect developed a resistance phenomenon. This was seen by the fact that conidia were trapped both in the cytoplasmic mass and strands which were seen in the gizzard and midgut. Furthermore, a thick peritrophic membrane developed around the epithelium of the midgut and did not allow the pathogen to attack intestinal cells.

#### ***Tests against Heterotermes indicola***

*Heterotermes indicola* workers when allowed to crawl on *A. flavus* culture for 10, 20 and 30 minutes showed about 11%, 7% and 16% mortalities at 24 hours following infection, respectively (Table-IV).

The regression curves related to the mortality of *H. indicola* are presented in Fig. 1. The value of slopes ranged from 3.5 to 3.9. The values of LT50 were calculated as about 64 hours, 48 hours and 37 hours; and the values of LT90 were about 148 hours, 100 hours and 79 hours when the termites were crawled for 10, 20 and 30 minutes on *A. flavus* cultures respectively (Table-V). In order to see the attack of *A. flavus* on *H. indicola* histopathological studies were carried out. The healthy workers of *H. indicola* were allowed to crawl for 30 minutes on 72 hours old culture of *A. flavus* and their histopathological studies were carried out at 120 hours following *A. flavus* infection. The studies showed that there were broken cuticular teeth of the gizzard. Conidia were seen concentrated on the broken portion of the cuticular teeth of the gizzard (Plate 3, Fig. a, b and c).



**Plate-2 Fig. a-f, *Aspergillus flavus*. a, hyphae and conidia attacking at cuticle and branching into body cavity of *Bifiditermes beesoni*, x 400. b, conidia surrounded by cytoplasmic strands in the lumen of gizzard, x400. c, conidia in the lumen of the gut and concentrated near epithelial cells of midgut, x400. d, a thick peritrophic membrane around midgut epithelium, and several conidia trapped in cytoplasmic strands, x 400. e, conidia in the lumen of the gut and concentrated near epithelial cells of midgut, x400. f, conidia in the lumen of the gut and concentrated near epithelial cells of midgut, x400. B, body cavity; C, cuticle; Cn, conidia; CS, cytoplasmic strands; CT, cuticular teeth; EP, epithelial cells; H, hyphae; L, lumen; NG, nerve ganglion; PM, peritrophic membrane.**

It appears that cuticular teeth of the gizzard were destroyed as a result of toxin produced by germinating conidia. In a few sections, the peritrophic membrane was seen fused with the regenerative cells of the midgut (Plate 3, Fig. d). In general, the midgut epithelial cells were seen in a disintegrated state, but the regenerative cells were not destroyed. It appears that peritrophic membrane did not allow germinating conidia to

attack regenerative cells.

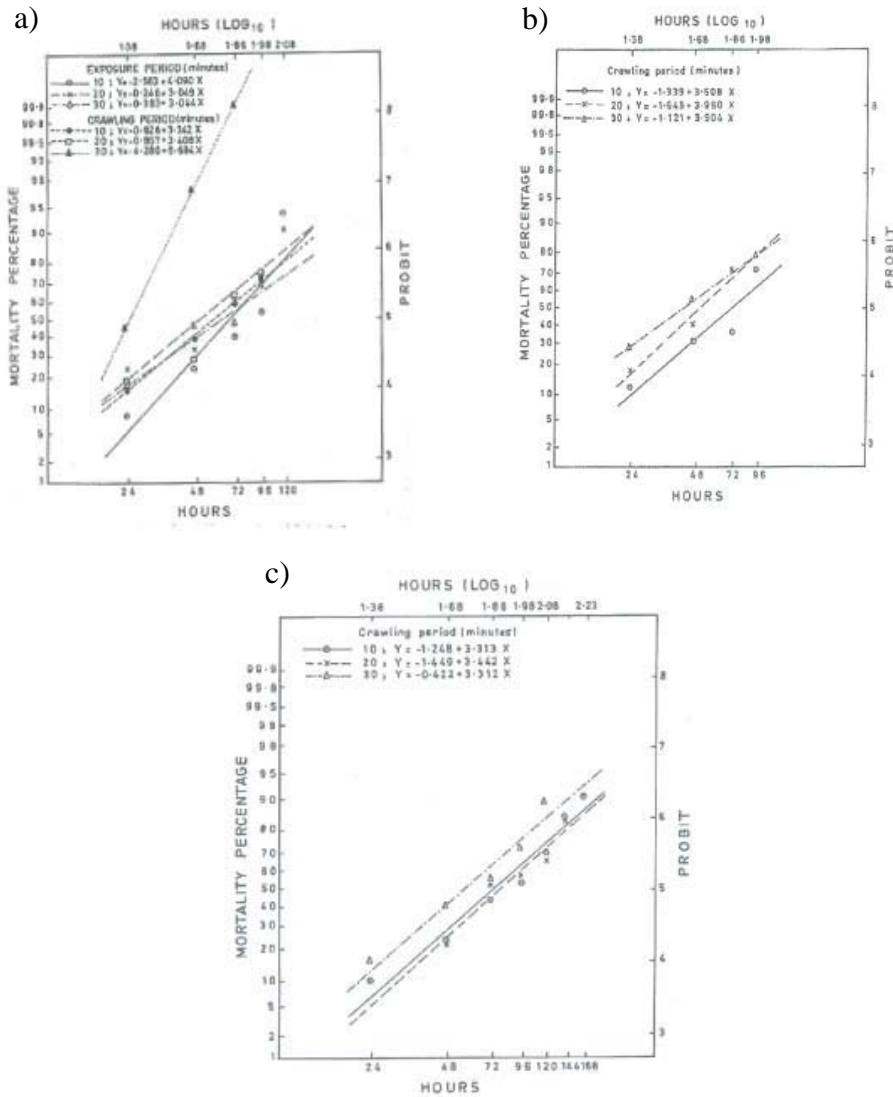


Fig. 1 Regression lines of mortality of a) *M. championi* b) *B. beelsoni* c) *H. indicola* after being exposed or crawled on the locally isolated *A. flavus* for a given period.

**Table III: Corrected mortality percentage of *B. beesoni* infected by 72 hours old culture of *Aspergillus flavus***

Hrs after infection	Gr. 1 Control	Gr. 2 Crawling 10min.	Gr. 3 Crawling 20min.	Gr. 4 Crawling 30min.
24	0.00	12.00	17.33	28.00
48	0.00	32.00	40.00	53.33
72	0.00	36.00	70.67	100.00
96	2.67	72.00	100.00	
120	2.67	100.00		

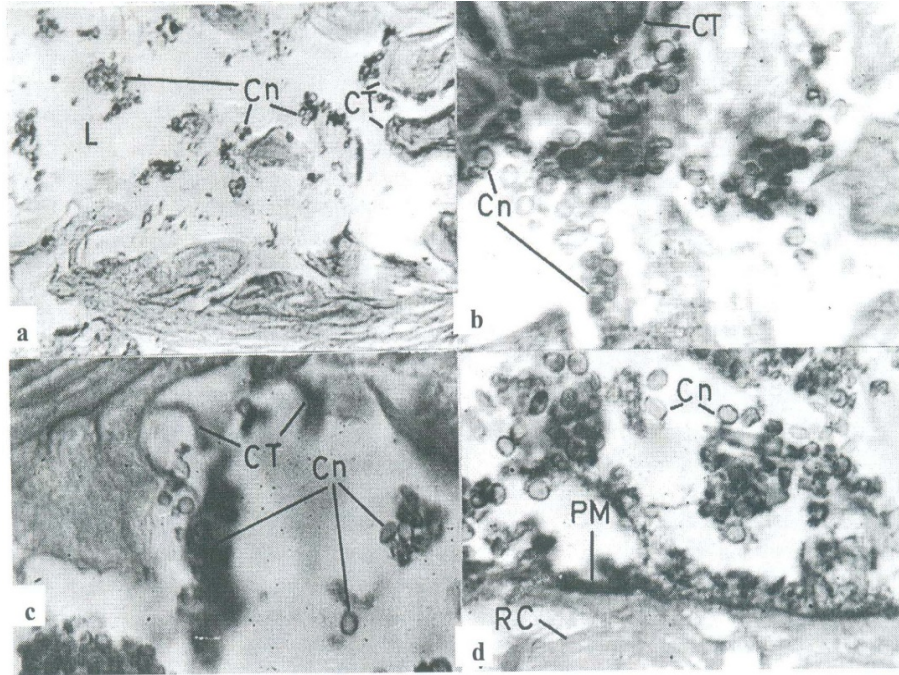
**Table IV: Corrected mortality percentage of *H. indicola* infected by 72 hours old culture of *A. flavus***

Hrs after infection	Gr.1 Control	Gr. 2 Crawling 10min.	Gr. 3 Crawling 20min.	Gr. 4 Crawling 30min.
24	1.33	10.67	6.67	16.00
48	2.67	24.00	24.00	42.67
72	6.67	40.98	53.33	56.69
96	8.00	54.51	61.02	71.91
120	9.33	60.21	64.69	89.65
144	9.33	85.31	83.85	100.00
168	9.33	91.30	100.00	
192	12.00	100.00		

**Table V:  $LT_{50}/LT_{90}$  and value of slopes of Regression lines of *M. championi*, *B. beesoni* and *H. indicola* infected by 72 hours old culture of *A. flavus***

Inoculation period (minutes)	*Microcerotermes		Bifiditermes		Heterotermes	
	$LT_{50}/LT_{90}$	$LT_{50}/LT_{90}$	$LT_{50}/LT_{90}$	$LT_{50}/LT_{90}$	$LT_{50}/LT_{90}$	Slope (b)
10	55.4/133.8	76.8/187.1	64.1/148.5	64.1/148.5	76.8/187.1	3.3±0.8
20	55.9/132.8	73.8/173.7	47.7/100.3	47.7/100.3	73.8/173.7	3.4±0.9
30	24.5/38.0	54.6/133.0	37.0/78.7	37.0/78.7	54.6/133.0	3.3±0.9

\*the data is taken from Table-II for comparison.



**Plate-3** Fig. a-d, *Aspergillus flavus*. a, conidia in the lumen and attacking the cuticular teeth of gizzard of *Heterotermes indicola*, x400. b, conidia are concentrated in gizzard, x1000. c, conidia showing corrosive action by dissolving the cuticular teeth of the gizzard, x 1000. d, conidia are surrounded by peritrophic membrane which is fused with regenerative cells, x 1000. Cn, conidia; CT, cuticular teeth; L, lumen; PM, peritrophic membrane; RC, regenerative cells.

## DISCUSSION

The green colored fungus was isolated and identified as *A. flavus*; soon its pathogenicity tests were carried out on the workers of *M. championi*. An analysis of the data pertaining to  $LT_{50}$ ,  $LT_{90}$  and value of regression slopes showed that *A. flavus* was fairly pathogenic to *M. championi*. The workers of *M. championi* were infected by two methods, the “exposure or overhead culture method” and “crawling or surface culture method”. Higher mortalities were obtained when termites were exposed to surface culture (crawling). Yendol and Rosario (1972) also obtained better results by the surface culture method.

Thus the pathogenicity of *A. flavus* was tested in the subsequent experiments only by crawling method. The comparative data of LT50, LT90 (Table V) of various species of termites show that workers of *M. championi* were most susceptible to *A. flavus* culture, as compared to *H. indicola* and *B. beesoni*. These findings support the view of Becker (1965) that differences exist in the action of strain of fungus to various species of termites.

When the workers of *M. championi*, *H. indicola* and nymphs of *B. beesoni* were crawled on *A. flavus* culture for a longer period (30 minutes) their mortality occurred in a shorter period of time. Yendol and Rosario (1972) described that effectiveness of an entomogenous fungus to kill its insect host depends greatly upon the doses of number of spores applied, number of germinating spores and the virulence of the fungal strain.

In the present study it appears that when termites were crawled on *A. flavus* culture for a longer period they came in contact with a large number of spores. Thus germinating spores caused quicker rate of mortality of termites. The rapid mortality could also be possibly explained by secretion of toxic material by the fungus (Prasertphon and Tanada, 1960; Yendol *et al.*, 1968; Yendol and Rosaria, 1972).

*Aspergillus flavus* Link has been reported to cause the infection in subterranean termites, *Reticulitermes virginicus* and *R. flavipes* (Beal and Kais, 1962). Exposure of termites to culture of this fungus resulted in mortality averaging 80% between 6 and 12 days. Sannasi (1968) also observed dead queen and drone of *Odentotermes obesus* and found *A. flavus* as the causative agent. Thus the present studies bring support to the earlier observations of Lund (1966) and others, that *A. flavus* is widely distributed as potential pathogen throughout the world and is considered in combating termites.

In the present study, mode of attack of pathogen on the host tissues and resistance mechanism of the host to the pathogen was observed. Histopathological studies showed that when the mode of entry of *A. flavus* in *M. championi* and *B. beesoni* was through integuments, its mycelia branched into the body cavity and attacked all the organs including the nerve cord (Plate 1, Figs. a, b, e and f.; Plate 2, Fig. a to f). When the conidia entered in *M. championi* and *B. beesoni* through oral route, they were seen trapped by cytoplasmic masses and strands developing in the gizzard and gut of the termite concerned (Plate 1, Fig. b

and d, Plate 2, Fig. d). The peritrophic membrane became thick in the gut of *B. beesoni* (Fig. d and e, Plate 3) and provided barrier to the attack of germinating conidia. In case of *H. indicola* the gizzard was attacked by conidia resulting in the destruction of cuticular villi (Plate 3, Fig. a to c). The peritrophic membrane acted as a barrier against the attack of conidia of *A. flavus* in the gut of *H. indicola* (Plate 3, Fig. d). In general, these observations brought the evidence that there is development of resistance phenomenon also against the attack of *A. flavus* in the gizzard and intestine of various species of termites. However, *A. flavus* will be considered as a potential pathogen for the control of termite in future; as various isolates of *M. anisopliae* and *B. bassiana* have potential for future use in control program (Delate *et al.*, 1995; Wells *et al.*, 1995; Jones *et al.*, 1996).

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