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Ultrastructural Studies to Establish the Mode of Action of *Pseudomonas fluorescens* against *Aspergillus flavus* using Scanning Electron Microscope

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A B S T R A C T

In Groundnut aflatoxin contamination is a major problem which affects the quality of the seed. These aflatoxins have carcinogenic, hepatotoxic, teratogenic and immuno-suppressive effects which were produced by *Aspergillus flavus* and *A. parasiticus*. For the management of aflatoxin problem in groundnut, use of plant growth-promoting rhizobacteria (PGPR) is a viable and sustainable option. Our present study is aimed at determining the mode of action of these PGPR (*Pseudomonas fluorescens*) using Scanning Electron Microscope (SEM). A superior *P. fluorescens* isolate (*Pf*7) identified from our studies in dual culture studies and *in vitro* seed colonization assay (IVSC) was screened against toxigenic isolate of *A. flavus* (AFT5B). In SEM, *Pf*7 exhibited both antibiosis and hyperparasitism. In antibiosis, deformation and coiling of hyphae of test fungus was noticed. Further, the mycelium appeared thread like, wrinkled and flaccid. Conidiophores are twisted near the zone of inhibition. In hyperparasitism, a white slimy growth of bacterial cells was seen on mycelia of test fungus. Further, structural disintegration of conidiophores and conidia of *A. flavus* was noticed. Overall, our results suggested the efficacy of *Pf*7 in reducing *A. flavus* infection and aflatoxin contamination through hyperparasitism and antibiosis.

Introduction

In India groundnut is an important oil seed crop which is a rich source of protein, dietary fiber, minerals, and vitamins (Ntare *et al.*, 2008). Among different biotic stresses in groundnut cultivation, aflatoxin contamination is an important one which occurs at both pre-andpost-harvest stages of the crop. These aflatoxins are produced by *Aspergillus flavus* and *A. niger*. It is a qualitative problem affecting grain quality andtrade (Waliyar *et al.*, 2008). Several management strategies have been attempted to minimize the aflatoxinproblem, in which biological control is a viable option. Of

different biocontrol agents, use of plant growth-promoting rhizobacteria (PGPR) is gaining momentum because of its plant growth promoting activity along with the suppression of *A. flavus* infection. *Pseudomonas fluorescens* is an important PGPR genus for the management of these aflatoxins in Groundnut.

For a PGPR isolate to be an effective candidate bio-agent against pre-harvest aflatoxin contamination, understanding its mechanism of action against *A. flavus* is mandatory. It is precisely at this stage, ultra-structural studies on the interaction between *P. fluorescens* and aflatoxin producing molds is required. Though, few reports on the interaction between these two microbes are available, comprehensive studies on the effect of antibiosis and hyper parasitism of a PGPR strain on *A. flavus* are still lacking. The present study therefore focused on documenting the effectiveness of elite PGPR isolate against *A. flavus* through scanning electron microscopy (SEM).

Materials and Methods

The present investigation was carried out with the facilities available International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. The scanning electron microscopy (SEM) work pertaining to the mode of action of plant growth-promoting rhizobacteria (PGPR) (specifically, *Pseudomonas fluorescens*) against *Aspergillus flavus* was carried out at Ruska Laboratories, Rajendranagar, Hyderabad of PV Narasimha Rao Telangana Veterinary University.

Antibiosis of *Pseudomonas fluorescens* on *Aspergillus flavus*

The potential *P. fluorescens* strain (*Pf*7) identified in dual culture studies and IVSC assay was used against the toxigenic *A. flavus* strain, AFT5b for establishing its mode of action. The antagonistic properties of *P. fluorescens* strain were studied using SEM (Gupta *et al.,* 2001; Weidenborner *et al.,* 1989; Gopalakrishnan *et al.,* 2015). The SEM facilities of Ruska laboratories were utilized in the present study. Plugs of mycelium (5 mm diameter) were cut from the edge ofan actively growing fungal colony on PDA with a No. 2 Cork borer, and one plug was placed in the center of each PDA plate (100 x 15 mm). Two parallel 3.5 cm long streaks of PGPR were then made 2 cm apart on opposite sides of the plug. The test fungus not co-inoculated with PGPR isolate was served as a control.

Later, the Petri dishes were incubated at 25°C for five days in the dark. Fungal mycelia growing towards the inhibition zone was processed for SEM by the following procedure. Agar discs of 1mm thickness were cut from mycelial growth of *A. flavus* near the inhibition zone and placed on cover glasses. For the fungal control the 1mm agar discs were sampled from the leading edge. The separated discs containing mycelia were washed with phosphate buffer, post fixed in 2% osmium tetroxide for 4 h and dehydrated using a graded series of ethanol.

The dehydrated samples were dried with critical- point liquid carbon dioxide as a transition fluid. The dried materials were adhered onto aluminum specimen mounts with double stick adhesive tape. The samples were later coated with gold-palladium in an automated sputter coater (JEOL JFC-1600) and examined with a scanning electron microscope (JEOL-JSM 5600) as per the standardized protocols at Ruska laboratories. Mycelial growth of *A. flavus*in control plates was also observed. The hyphal deformities near the zone of inhibition were recorded and compared with that of control plates.

Hyperparasitism of *Pseudomonas fluorescens* strain and *Aspergillus flavus*

The *A. flavus*culture was multiplied on PDA at 28ºC for 36 h under dark conditions. Eight mm diameter plugs that were cut from the leading edge of *A. flavus* culture were later sprayed with *P. fluorescens* (*Pf*7) at a concentration of 1 x 10^8 CFU ml^{-1} and incubated for three days at 28ºC. Fungal discs sprayed with sterile distilled water were served as the controls. Discs of fungal mycelium were prepared for SEM (Ziedan *et al.*, 2008).

Samples were washed with phosphate buffer, post fixed in 2% osmium tetroxide for 4 h and dehydrated using a graded series of ethanol. The dehydrated samples were dried with critical- point liquid carbon dioxide as a transition fluid. The dried materials were adhered onto aluminum specimen mounts with double stick adhesive tape. The samples were later coated with gold-palladium in an automated sputter coater (JEOL JFC-1600) and examined with a scanning electron microscope (JEOL-JSM 5600) as per the standardized protocols at Ruska laboratories. The occurrence of morphological changes in the hyphae of *A. flavus* was recorded and compared with that of healthy hyphae in control plates.

Results and Discussion

From the ten strains of *P. fluorescens*, the results of dual culture studies have shown that *Pf*7 was highly effective in reducing the mycelial growth of *A. flavus*. Further, in IVSC assays, the *Pf*7 strain could inhibit the seed colonization and aflatoxin contamination by *A. flavus*. Hence, this potential strain, *Pf*7 was selected to study its mode of action on the toxigenic *A. flavus*strain (AFT5b). The scanning electron microscopic image of the *P. fluorescens* (*Pf*7) is depicted in Figure 1 (a&b).

Antibiosis of *Pseudomonas fluorescens* on *Aspergillus flavus*

SEM studies on the antibiosis of *Pf*7 strain on toxigenic *A. flavus* (AFT5b) revealed that there was a definite inhibition of *A. flavus* hyphae. Clear deformation and coiling of hyphae near the zone of inhibition between test fungus and bio-agent was noticed. Marked differences were noticed in the structure of mycelia, conidiophore and conidia near the zone of inhibition and in control plates (healthy). The mycelial structure of *A. flavus* in the control plates was tubular, turgid, and the hyphae were linearly elongated. Further, mycelia grew densely in various directions, with numerous conidia on their surface (Fig. 2a&b). In contrast, the mycelium in the treatment (near the zone of inhibition) appeared thread like, wrinkled and flaccid. Further, the hyphae were seen shriveled, with a clear reduction in the hyphal branching (Fig. 3 a&b). The conidiophores of *A. flavus* near the zone of inhibition in treatment were seen twisted and distorted (Fig. 4 a&b). However, such coiling and distortions of conidiophores of healthy *A. flavus* in control plates were not seen. (Fig. 5 a&b) Overall, the SEM studies revealed the effect of antibiosis of *P. fluorescens* (*Pf*7) on *A. flavus*.

Hyperparasitism of *Pseudomonas fluorescens* strain and *Aspergillus flavus*

The studies on effect of hyperparasitism of *P. fluorescens* (*Pf*7) on the mycelia of *A. flavus* (AFT5b) revealed the presence of white slimy growth of bacterial cells on the *A. flavus* mycelia (Fig. 6). Further, the bacterial cells were seen engulfing the conidia of *A. flavus* (Fig. 7). Observations also revealed coiling and shriveling of conidiophores, deformities in conidia and conidiophores of *A. flavus* due to hyperparasitism (Fig. 8). Besides, there was also structural disintegration of conidiophores

of *A. flavus* due to hyperparasitism. Rarely, breakdown of conidiophores were also noticed due to hyperparasitism (Fig. 9). On the other hand, conidiophore and conidia were normal, without any shrinking, shriveling and deformities in control plates.

Ultrastructural studies on interaction between bio-agent and a test pathogen assumes significance in order to understand the nature of antagonism that is exhibited by bio-agent. However, SEM studies between PGPR and the aflatoxin producing mold, *A. flavus* are scanty. Mostly, SEM studies focused on understanding the structure of these aflatoxigenic molds and their identification up to species level and characterization (Rodriguez *et al.,* 2007). Further, these SEM studies also are used to confirm the seed borne nature of *A. flavus* in groundnut. For example, in a study by Achar *et al.,* (2009), it was observed that mycelium of *A. flavus* was seen established in the host tissues both intercellular and intracellularly. Further, continuous branching of young hyphae was seen. Mycelial structures were also detected in xylem vessels of roots, confirming its systemic infection. Other SEM studies are focused on confirming the biological synthesis of nano-particles such as silver by *A. flavus* (SubhaRajam *et al.,* 2013). Colonization of rhizosphere of crop plants by PGPR strains and thereby plant disease control is another area where SEM studies are used. For example, in a study on the effects of fluorescent *Pseudomonads* (CW2 isolate) in cucumber rhizosphere, SEM results indicated that the frequency of PGPR (CW2) colonization was higher in cucumber roots infested with fungi than in healthy roots (Salman *et al.,* 2013). In another study by SEM, it was reported that the endophytic PGPR isolates such as *Bacillus subtilis* and *P. fluorescens* were able to colonize cortex tissue of groundnut roots. Further, the ultrastructural studies using transmission electron microscopy (TEM) of groundnut root tissues revealed that when these endophytic PGPR strains were inoculated onto root surfaces, the plant defense reactions were activated (Ziedan, 2006).

Fig.1 (a&b) Scanning electron photomicrograph of the *Pseudomonas fluorescens* (*Pf7*) showing rod shaped bacterial cells

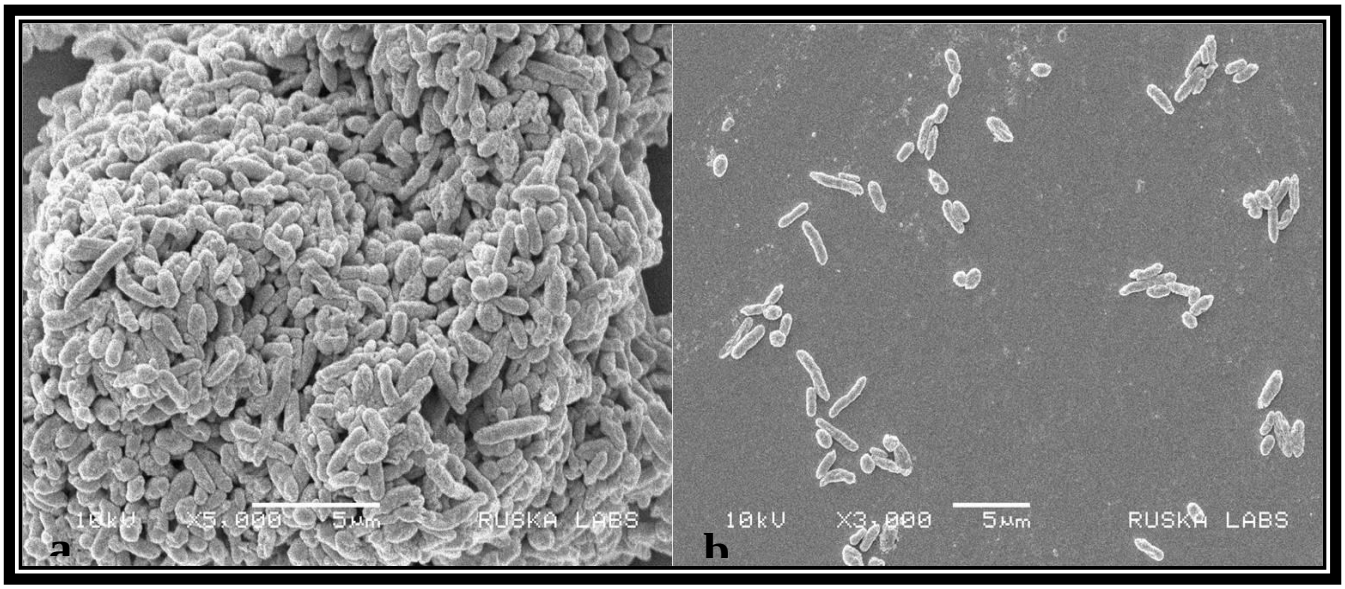


Fig.2 (a&b) Scanning electron photomicrograph of *Aspergillus flavus* (AFT5b) in Control plates (healthy) showing dense, tubular, linearly elongated hyphae spread in various directions with numerous conidia

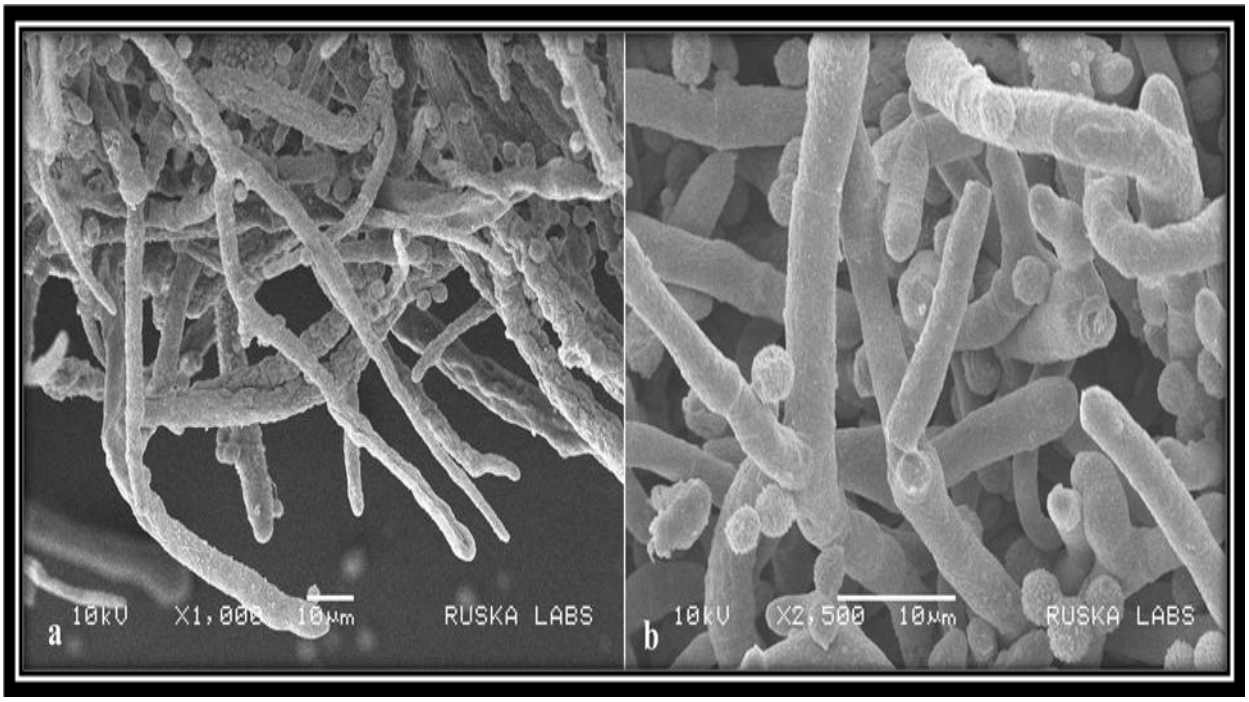


Fig.3 (a&b) Scanning electron photomicrograph showing the effect of antibiosis of *Pseudomonas fluorescens* (*Pf7*) on *Aspergillus flavus* (AFT5b). (The hyphae of *A. flavus* appear as thread like, wrinkled, flaccid, shriveled and with reduced hyphal branching)

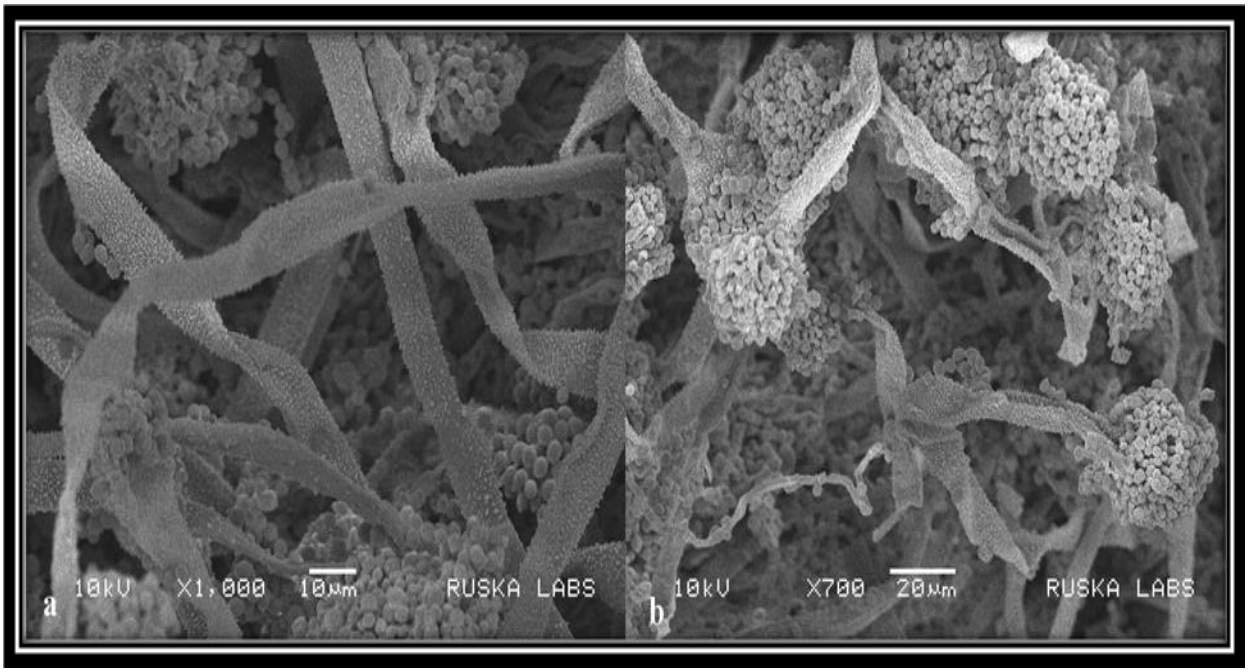


Fig.4 (a&b) Scanning electron photomicrograph showing the effect of antibiosis of *Pseudomonas fluorescens* (*Pf7*) on *Aspergillus flavus* (AFT5b). (The conidiophores near zone of inhibition appear as twisted and distorted)

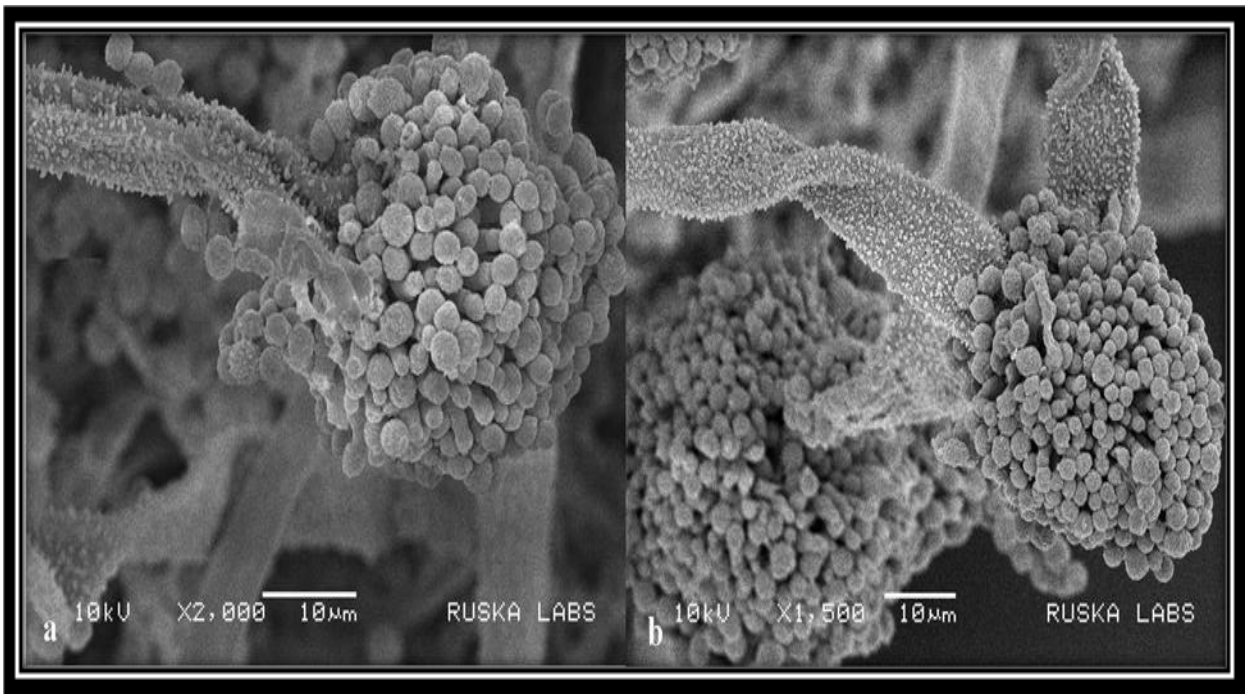


Fig.5 (a&b) Scanning electron photomicrograph of conidiophore of *Aspergillus flavus*, AFT5b with numerous conidia in control plates (healthy)

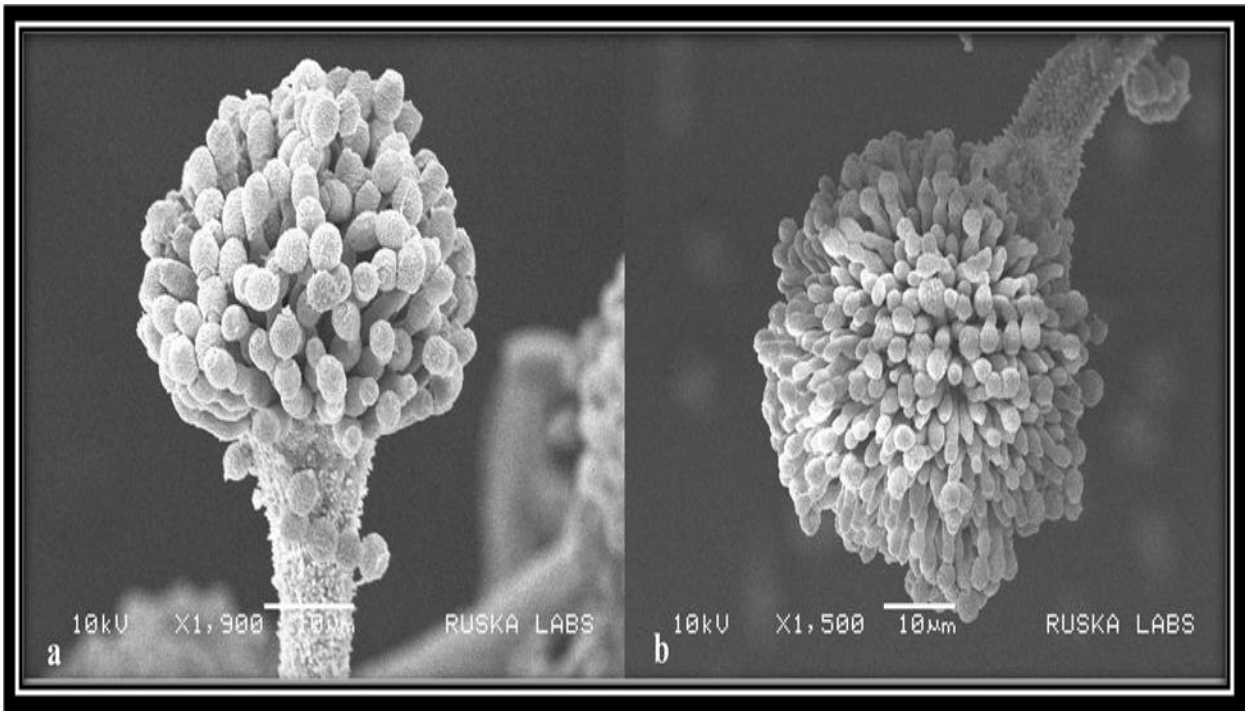


Fig.6 Scanning electron photomicrograph showing the slimy growth of *Pseudomonas fluorescens* (*Pf7*) on the hyphae of *Aspergillus flavus* (AFT5b) as a result of hyperparasitism

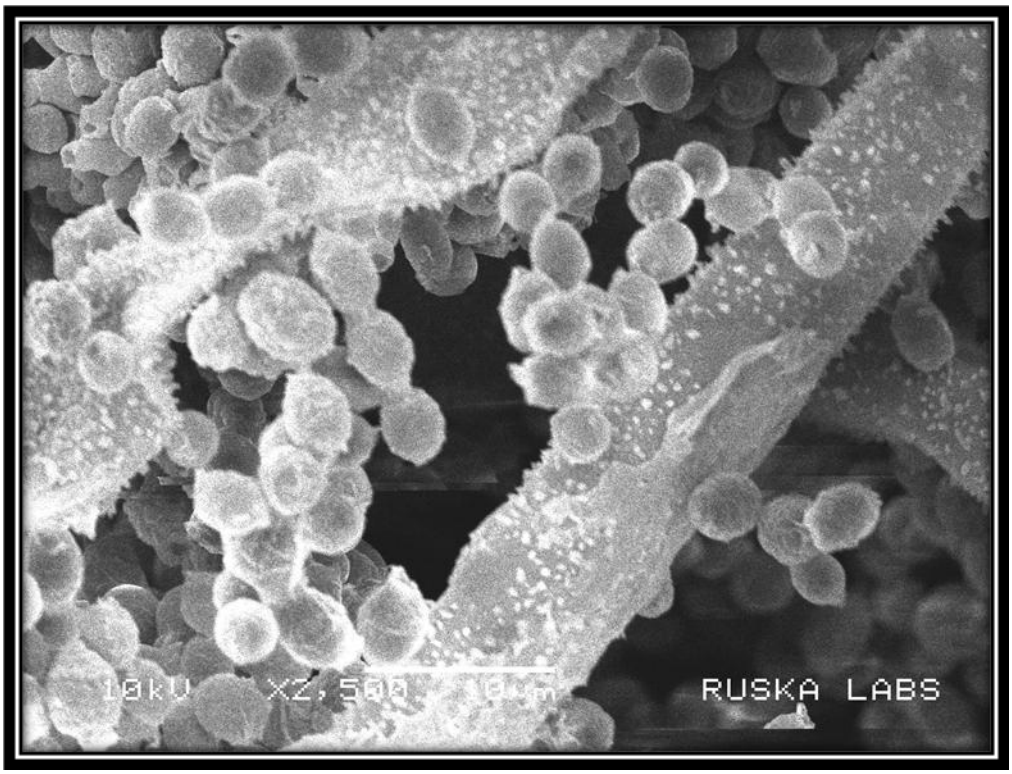


Fig.7 (a&b) Scanning electron photomicrograph showing the engulfing of *Aspergillus flavus* (AFT5b) hyphae by *Pseudomonas fluorescens* as a result of hyperparasitism

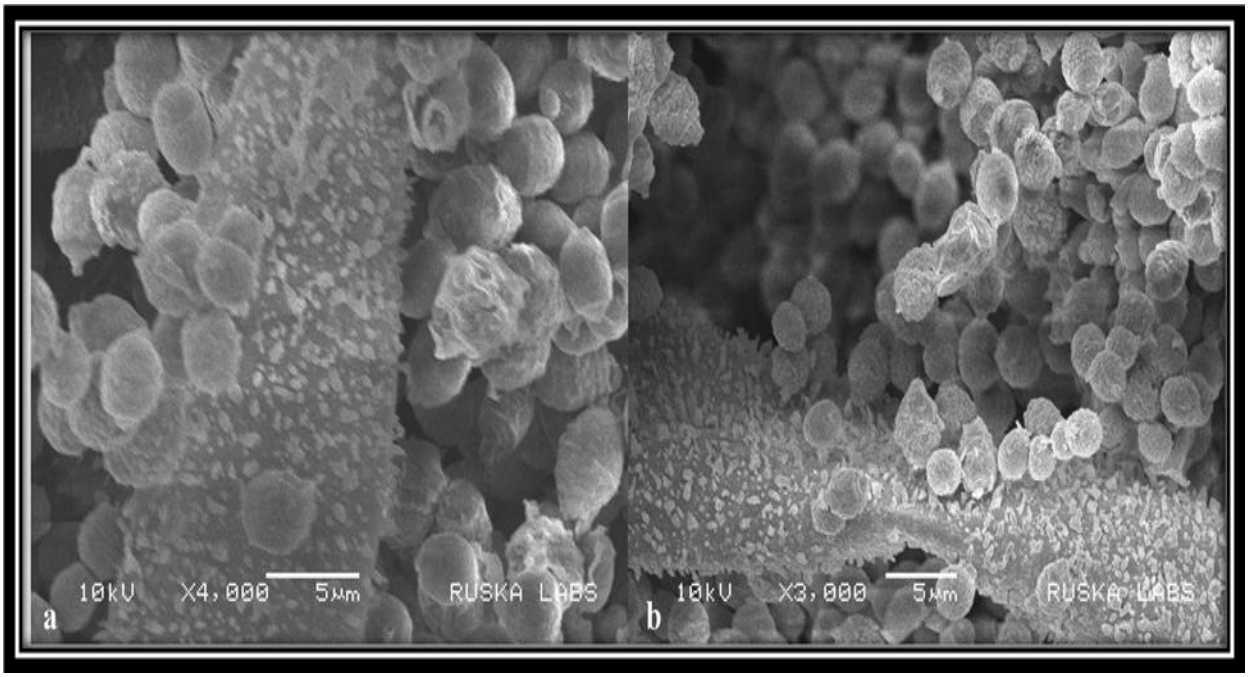


Fig.8 Scanning electron photomicrograph showing the effect of hyperparasitism of *Pseudomonas fluorescens* (*Pf*7) on *Aspergillus flavus* (AFT5b) (Shriveled, deformed conidiophores and conidia)

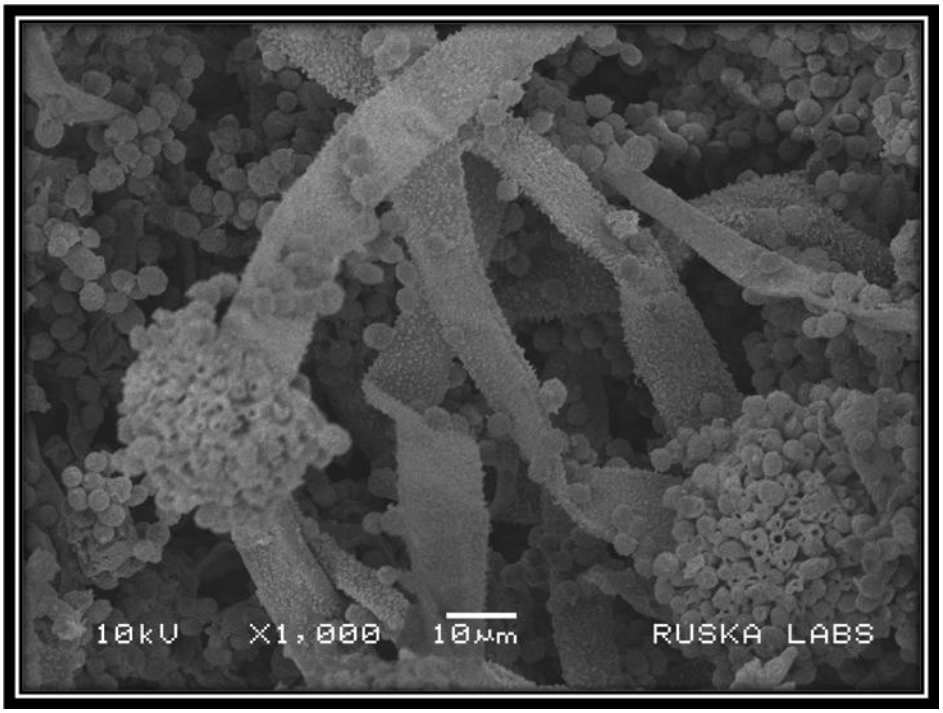
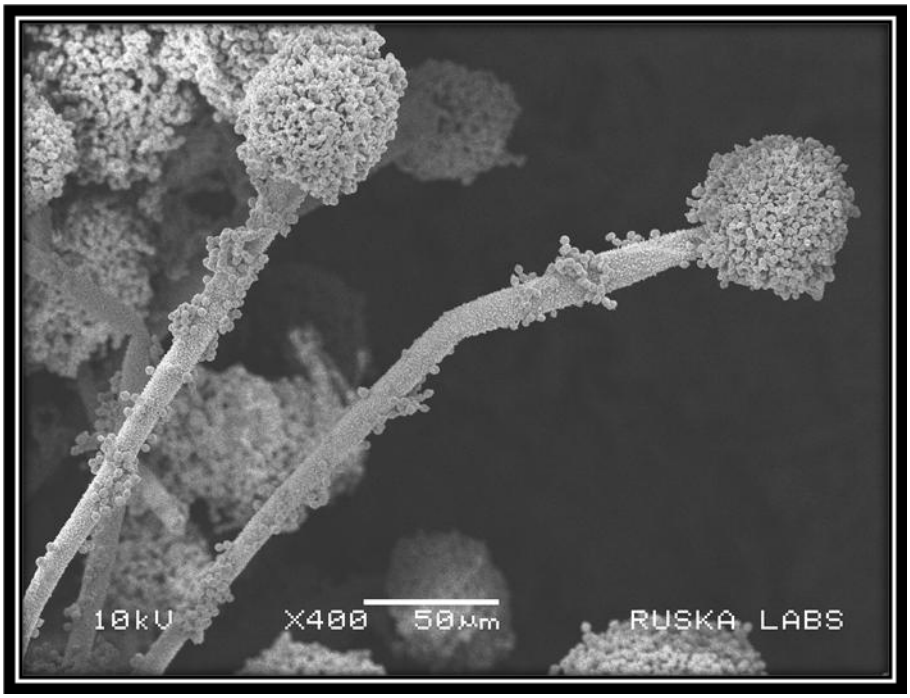


Fig.9 Scanning electron photomicrograph of *Aspergillus flavus* conidiophore showing hyphal breakdown due to hyperparasitism reaction



Only few reports are available on establishing the antifungal activity of microbes against *A. flavus*. For example, SEM studies have confirmed the antifungal activity of glycolipids produced by *Rhodococcus erythropolis* on *A. flavus* (Abdel-Megeed *et al.,* 2011). Specific studies on the antagonism of PGPR strains on *A. flavus* are focused on post-harvest losses in agricultural commodities.

For example, use of fungal cell wall-degrading enzymes produced by biological control agents against *A. flavus* is one area that is confirmed using SEM studies. In a study by Akocak *et al.,* (2015), the antagonistic effects of PGPR strains such as *P. fluorescens* (PB27) and *Bacillus cereus* (B1) against aflatoxigenic *A. flavus* were investigated.

The SEM results indicated that, when the test fungus was exposed to chitinolytic PGPR strains, PB27 and B1, the test fungus, *A. flavus*has shown numerous ultrastructural morphological changes during spore germination and mycelial growth.

However, several reports have established the antagonism of PGPR strains on other plant pathogens. The antagonistic effects of *P. fluorescens* (CW2 isolate) in cucumber rhizosphere on important root pathogens such as *Pythium ultimum* and *Rhizoctonia solani*are well established. When cucumber roots were drenched with CW2 strain, it caused irregular and abnormal fungal growth.

The SEM studies further confirmed the swellings and shrinkages of *P. ultimum* and *R. solani* hyphae (Salman *et al.,* 2013). In a study by Vijay Krishna Kumar *et al.,* (2013), it was observed that the PGPR strain, *B. subtilis* MBI 600 (commercially available as Integral®) was highly antagonistic to soilborne pathogen of rice, *Rhizoctonia solani*, the causal agent of sheath blight disease. Ultrastructural studies on the interaction between sheath blight pathogen and MBI 600 indicated that the bio-agent has exhibited both antibiosis and hyperparasitism. The bio-agent caused loss of structural integrity, shriveling, abnormal coiling and lysis of *R. solani* hyphae due to antibiosis and hyperparasitism. Further, maceration and fragmentation of inner walls of sclerotia of sheath blight pathogen were observed when the sclerotia were treated with commercial formulation of MBI 600.

In our studies, the *Pf*7 strain exhibited higher degree of antibiosis on *A. flavus*. Further, with one week of incubation after spraying of *Pf*7 on test fungus, the conidiophores were structurally disintegrated, coiled and shriveled with deformities.

Further, breakdown of conidiophores due to engulfing by bacterial slimy growth was noticed, indicating hyperparasitism. Future investigations through SEM are necessitated to investigate the mode of action of *Pf*7 in inhibiting *A. flavus* entry on seeds. Further, SEM on spermoplane and rhizosphere colonization of *Pf*7, besides its endophytic nature in groundnut if any has to be investigated.

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