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Isolation of entomopathogenic fungus, *Aspergillus flavus* and its efficacy against cotton mealy bug (*Phenacoccus solenopsis*)

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ABSTRACT

Phenacoccus solenopsis is commonly known as cotton mealy bug is a harmful pest that affects cotton (*Gossypium* spp.) plants as well as ornamental plants and other agricultural products due to its ability to feed on a wide variety of plants. The experiment was conducted during 2023 at Thiagarajar College, Madurai, Tamil Nadu to investigate the potential of the entomopathogenic fungus *Aspergillus flavus* as a control method for the 3rd nymphal instar of *P. solenopsis* in the laboratory assay. The entomopathogenic fungi were isolated from soil by *Galleria* bait method. The pathogenicity of the fungi was evaluated through dipping bioassays using three different concentrations of conidial suspensions (1×10^6, 1×10^7 and 1×10^8) and a negative control of 0.05% Tween 80. The experiments were repeated three times, each with 25 insects. The results showed that *A. flavus* was effective in killing the pest, with a mortality rate of 89.3% after 120 h. The pathogenicity of *A. flavus* was further analyzed through histological studies. The GC-MS analysis of the ethyl acetate extract of *A. flavus* confirmed the presence of insecticidal compounds such as pthalic acid and dibutyl phthalate. Results indicated that *A. flavus* shows promise as a biocontrol agent against *P. solenopsis* and could be considered for use in field applications.

Keywords: *Aspergillus flavus,* Biological control, Entomopathogenic fungi, Insect pest, *Phenacoccus solenopsis*

The cotton mealybug, *Phenacoccus solenopsis* is a notorious insect pest of cotton plants (Tong *et al.* 2019). The cotton mealy bug were reported as a serious and invasive threat to cotton crops in India, causing significant economic losses in Asian countries (Vennila *et al.* 2010). *Phenacoccus solenopsis* has a wide range of adaptation to different climatic conditions. It is a polyphagous insect feeding on diverse group of plants including weeds, horticultural and agricultural crops (Wang *et al.* 2023) that feeds on plant fluids, leading to wilting, stunting, and death of the affected plants. This feeding also produces waxy secretions that obstructs the entry of chemical pesticides and reduces the effectiveness of chemical control (Ulusoy *et al.* 2022). *P. solenopsis* has been identified as pest of 154 host-plant species out of which 20 field crops, 64 weeds, 45 ornamental plants and 25 shrubs and trees, belonging to a total of 53 plant families (Noureen *et al.* 2016). Wide range of mealy bug species developed resistance against chemical pesticides. Nymphal instars of *P. solenopsis* developed resistance to various insecticidal compounds including pyrethroids, organophosphates and neonicotinoids (Ahmad and Akhtar 2016). Nagrare *et al.* (2020) reported the development of resistance against insecticides among the population of *P. solenopsis* in Maharashtra, India. Emergence of resistance against insecticides in cotton mealy bug is a critical issue in pest control, which may lead to ecological and socio-economic problems (Afzal and Shad 2015).

To reduce reliance on these harmful chemicals, alternative control methods must be developed (Sandhu *et al.* 2012). Identification of biological control agents is the crucial process in the implementation of the biological pest management strategies. Entomopathogenic fungi are diverse group of fungal species. They are ecofriendly and non-toxic fungi used for biological management of insect pests of agricultural crops (Araujo and Hughes 2016). Several species of entomopathogenic fungi was recognized as important biocontrol agents, to protect against various agricultural insect pests (Cuthbertson and Audsley 2016). The common source of entomopathogenic fungi are insect cadavers and rhizosphere soil (Singh *et al.* 2016). The present study was aimed to isolate the entomopathogenic fungi from soil sample and investigate their pathogenicity against 3rd instar nymphs of *P. solenopsis*. The lethal concentration 50 and lethal time 50 were evaluated to assess the effectiveness of the isolated fungi against the mealybug.

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MATERIALS AND METHODS

Rearing of P. solenopsis: The experiment was conducted during 2023 at Thiagarajar College, Madurai, Tamil Nadu. *Phenacoccus solenopsis* were reared on potato sprouts. Potato tubers were grown in plastic trays. Water was sprinkled daily to maintain wetness. After the sprouting of potatoes adult females of *P. solenopsis* collected from the field were transferred onto each sprout with the aid of camel hair brush. Third instar nymphs were collected and used for further analysis, as they represented an optimal stage of development and size.

Isolation of fungal cultures: Soil samples were collected from agricultural fields of Otthakadai (9.9584°N latitude, 78.1877°E longitude), Madurai, Tamil Nadu. The cropping history of field indicates a rotation of paddy and cotton. The 4th instar larva of wax moth, *Galleria melonella* were soaked in 56°C water for 15 sec. Soil sample was taken in a plastic container with perforated cover and the wax moth larvae were placed on the surface of soil. The soil sample was observed at regular time period. The dead larvae were removed and incubated for the development of mycelium. The fungi were isolated from the infected larvae showing mycelial growth on the external surface of body. The isolated fungi were sub-cultured in PDA (Zimmermann 1986) (Fig. 1).

Preparation of medium for culturing fungi: Potato dextrose agar was used to culture the fungi. 200 g of potatoes were diced and boiled in 1000 ml of distilled water. Potato extract was filtered through muslin cloth. Agar and 20 g of dextrose was added to the extract and transferred into culture flasks, then sterilized in an autoclave at 15 lbs pressure for 30 min.

Cultural characterization: Pure culture plate was observed for morphology characteristics of the isolates including colour and texture. For cultural characterization of isolated fungi, slide culture technique was adopted. A 1 cm block of potato dextrose agar was cut with a sterile scalpel. The agar block was then transferred to the glass slide. The fungal spores were inoculated on the four sides of the cover slip with the help of a sterile needle and it was placed over the agar block. The slide was placed inside a petri dish with a sterile filter paper at the bottom. In order to maintain the moisture, sterile distilled water was added to the filter paper at the base of the culture plate and incubated for five days at 27°C. After the fungal growth the cover slip was removed and stained with Lactophenol cotton blue and viewed under the microscope at 40X magnification. The structure of conidia and conidiophores of the fungi were also examined by Scanning Electron Microscopic analysis.

Fig. 1 *Galleria* Bait technique for the isolation of entompathogenic fungi from soil, mycosis of *Aspergillus flavus* on the larvae of *Galleria mellonella* after 14 days of baiting.

Isolation of DNA: The Fungal DNA was isolated using using NucleoSpin® Plant II Kit (Macherey-Nagel). About 100 mg of the fungal mycelium was homogenized using liquid nitrogen and 4000 microlitres of buffer PL1 was added and vortexed for 1 min. 10 µl of RNase A solution was added and incubated at 65ºC for 10 min. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 × g for 2 min. The flow through liquid was collected and the filter was discarded. 450 µl buffer PC is added and mixed well. The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 min and the flow through liquid is discarded. 400 µl of PW1 buffer is added to the column, centrifuged at 11000 × g for 1 min and flow though liquid is discarded. Finally 200 µl of PW2 is added and centrifuged at 11000 × g for 2 min. The column is transferred to a new tube and 50 µl of buffer PE is added and incubated at 65ºC for 5 min. The column is then centrifuged at 11000 × g for 1 min to elute the DNA. The eluted DNA was stored at 4ºC.

Polymerase chain reaction: The isolated DNA was amplified by Polymerase chain reaction. Molecular Identification of the fungal sequence was analyzed with Internal Transcribed Spacer (ITS) with primers ITS-F1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4R (5′-TCCTCCGCTTATTGATATGC-3′), primers. The PCR amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). The PCR reaction mixture included 5 µL of master mix, 4 µL of distilled water, 0.25 µL of forward primer, 0.25 µL reverse primer and 1 µL of DNA. PCR amplification profile includes, Initial denaturation of 98ºC for 30 sec, 40 cycles of 98ºC for 5 sec, 58ºC for 10 sec, 72ºC for 15 sec respectively, followed by final extention at 72ºC for 60 sec.The sequencing process was done using Big Dye Terminator v3.1 Cycle sequencing Kit. The quality of DNA sequence was determined using Sequence Scanner Software v1 (Applied Biosystems). The alignment and editing of sequences were done using Genius Pro v5.1.

Preparation of crude fungal extract: The fungi were cultured on potato dextrose broth and incubated for two weeks at 25°C. The broth culture was filtered through filter paper. The culture filtrate was separated and subjected to Ethyl acetate solvent extraction. The culture filtrate was mixed with equal volume ethyl acetate and mixed for 30 min. The extraction resulted in the formation of two separate, immiscible phases and the solvent phase was separated from aqueous phase by separating funnel. Then the metabolite was extracted by removing the solvent in a rotary evaporator at 40ºC. The fungal metabolite extract was stored at 4°C.

GC MS analysis: The sample was analyzed in GC Clarus 500 Perkin Elmer with Turbomass 5.2 software. 2 µl of sample was taken and introduced through an all-

glass injector operating in the split mode, with Helium as the carrier gas with a flow rate of 1.2. The HP-5 fused silica capillary column with a length of 30 m, 25 µm of film thickness and 0.2 mm ID. The library search of the compounds were done in NIST mass spectral library (Arul Pamila and Karpagam 2018).

Preparation of conidial suspension: Pure culture of the fungi was grown on Potato Dextrose Agar for 14 days. The spores were separated from the culture plate by scraping over the surface of the mycelium using a spatula. The fungal spores were transferred into 50 mL of Tween-80 (0.05%) solution. The spore suspension was filtered with cheese cloth to remove the mycelium. The spore concentration of 1×10^6, 1×10^7, and 1×10^8 were determined using a Neubauer hemocytometer.

Efficacy test: The efficacy of the fungal isolate was examined by dipping bioassay. The 3rd nymphal instars of mealy bugs were treated with spore suspension to analyze the efficacy of the fungal spore. Three different concentrations (1×10^6, 1×10^7 and 1×10^8 spore/mL) of spore suspension were prepared and 0.05% Tween 80 was used as control. About 25 nymphs were used for each treatment. The experiment was replicated thrice. The treated nymphs were observed daily and number of dead insects was recorded. The dead nymphs were incubated in moist condition to facilitate the development of mycosis. The percentage of mortality was calculated by the following formula:

$$\text{Percent mortality} = \frac{\text{Number of dead insects}}{\text{Number of treated insects}} \times 100$$

Histological test: Nymphs of *P. solenopsis* which are treated with conidial suspension of *A. flavus.* The infected insects and untreated insects (control) were subjected to sectioning and staining. Transverse sections of 2–4 µm thickness were made using a microtome. The sections were stained with Hematoxylin-eosin stain. Sections were treated with alcohol to remove moisture followed by xylene. The sections were mounted with DPX adhesive resin.

Statistical analysis: Lethal concentration (LC 50) and Lethal time (LT 50) of the fungal isolates was determined by Probit analysis. The significance of the study was statistically tested by Simple linear regression analysis using Graph pad prism 5 software.

RESULTS AND DISCUSSION

The cotton mealy bug, *Phenacoccus solenopsis* is a highly destructive pest of economically important crops. The control of mealy bugs has been largely dependent on chemical pesticides, despite concerns over their environmental impact. The challenge of reducing chemical pesticide usage and overcoming pesticide resistance in mealy bugs demands the exploration of alternative, sustainable management approaches. This study evaluated the insecticidal potential and histological effect of locally isolated entomopathogenic fungus *A. flavus* against third nymphal instars of *P. solenopsis*. The entomopathogenic fungus was isolated from soil. The isolated culture was identified as *Aspergillus flavus* by cultural and molecular characterization. The cultural characteristic of fungi was determined by microscopic observation and SEM analysis. *Aspergillus flavus* is a sporophytic haploid filamentous fungus which is yellow-green in colour with powdery conidia. The morphology of fungal colonies were flat at the edges and raised in the center. The fungal colonies exhibited a biseriate arrangement composed of philiades radiating outward in all directions from metulae which is supported by globose vesicles. The conidiophore composed of inflated spherical shaped spores with thin walls and rough surface morphology. Conidia were arranged in a chain like formation and exhibited a basipetal arrangement with the youngest conidia forming at the base and the matured conidia at the top (Fig. 2).

The molecular identification of the isolated fungi was carried by ITS sequencing. The obtained sequence was aligned by BLAST. The sequence of the isolated fungi showed 99.62% similarity with *Aspergillus flavus.* The nucleotide sequence was submitted in GenBank (OP061005).

The compounds found in the ethyl acetate extract were analyzed through GC-MS analysis. The results indicated the presence of dibutyl phtalate and pthalic acid, di (oct-3-yl) ester (Fig. 3). While, dibutyl phthalate compounds have raised concerns regarding the higher persistence in the environment, their mode of origin alters the level of toxicity and persistence. Dibutyl pthaltes and their derivatives produced by fungi are comparatively less persistent and easily degradable (Tian *et al.* 2016). Entomopathogenic fungi produce bioactive compounds like metabolites which affect the immune functions and manipulate the cellular

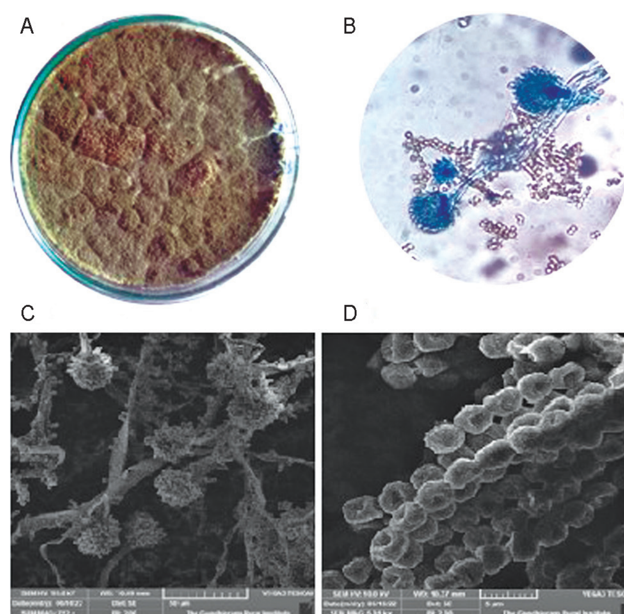

Fig. 2 (A) Pure culture of *Aspergillus flavus* cultured on PDA medium; (B), Microscopic observation of conidia and conidiophores of *Aspergillus flavus;* (C and D), Scanning electron microscopic (SEM) observation of conidiophores and conidia of *Aspergillus flavus*.

metabolism of host insects (Ortiz-Urquiza and Keyhani 2013). Soil fungi are active producers of biologically active compounds which are toxigenic against arthropods. Numerous compounds were identified from *Aspergillus* spp., maximum of which exhibited insecticidal property (Berestetskiy and Hu 2021).The fungal toxins which are produced as secondary metabolite exhibit lethal activity against insect pests and plays significant role in pest control (Kaur *et al.* 2021). Fungal derived secondary metabolites of *Aspergillus* spp. are highly virulent and effective against all developmental stages of insect life cycle (Arunthirumeni *et al.* 2023).

GC MS analysis confirmed the presence of phthalic acid and dibutyl phthalate in the crude extract of *A. flavus*, both of which have insecticidal properties that contribute to its efficacy. Adsul *et al.* (2012) found that the di-n-butyl phthalate extracted from the leaves of *Ipomea carnea* was effective against mosquito larva. Xu and He (2010) reported the insecticidal nature of phthalates which could be used as an insect repellent. Ullah *et al.* (2014) reported that phthalic acid showed significant toxicity against *G. mellonella* larvae and he also determined the inhibitory effects of pthalic acid on the host immune responses. Rajamanikyam *et al.* (2017) inferred that dibutyl phthalate isolated from *Brevibacterium mcbrellneri* have mosquito larvicidal activity with significant acetylcholinesterase inhibition and cytotoxicity.

The insecticidal potential of *A. flavus* was studied against the 3rd instar nymph of *P. solenopsis* using three different concentrations of conidia (1×10^6, 1×10^7 and 1×10^8 spore/mL). The percentage of mortality was calculated after five days of treatment (Fig. 4). The rate of mortality increased proportionally with time and concentration of fungal spores. The highest mortality of *P. solenopsis* (89.3%) was noted at a concentration of 1×10^8 spore/mL after 120 h of exposure. Results showed that the isolate exhibited a significant insecticidal potential against the *P. solenopsis* at a concentration of 1×10^8 spore/mL.

The LC 50 and LT 50 were calculated by Probit analysis. The estimated lethal concentration required for killing 50% of the nymph of *P. solenopsis* treated with *A. flavus* is 6.02 $\times 10^6$ over 120 h of treatment. LT50, the time required for killing 50% of the nymph at a concentration of 10^8 conidia/ml is 60.3 h, under laboratory conditions. Results showed that the mortality resulted by *A. flavus* was found to be significant ($p<0.05$) ($R^2 = 0.99$).

Our results are in consistent with previous reports stated by Bazazo *et al.* (2019) who found that *A. candidus* was an effective biological control agent against *P. solenopsis* with an overall mortality of 75.00% in the laboratory. The insecticidal potential of *A. flavus* has been reported against various insect pests. Kaur *et al.* (2021) have reported the oxidative stress causing potential and immunosuppressant nature of *A. flavus* against *Spodoptera litura*. The effect of *A. flavus* on reproductive activities of *Hieroglyphus oryzivorus* was revealed by Kumar *et al.* (2015). Nawaz and Freed (2021) stated that, *Beauveria bassiana, Metarhizium anisopliae* and *Isaria fumosorosea* were effective against the 2nd nymphal instar of *P. solenopsis*. Khanzada *et al.* (2021) found that *B. bassiana* and *M. anisopliae* were highly effective against *P. solenopsis. Aspergillus flavus* has the ability to induce oxidative stress in host insects by modifying the level of antioxidant enzymes during mycosis (Jayanthi *et al.* 2015, Karthi *et al.* 2018).

The primary means of entry for entomopathogenic fungi into the host is by cuticle penetration. EPF is the primary source of pathogen among sucking insect pests, such as aphids these insects cannot ingest other pathogens (Sharma *et al.* 2023). In the current study, the dead insects after the treatment with fungal suspension were incubated in moist condition. The death of the insects due to fungal infection was further confirmed by development of fungal conidia. Post-treatment observations revealed mycelial growth of

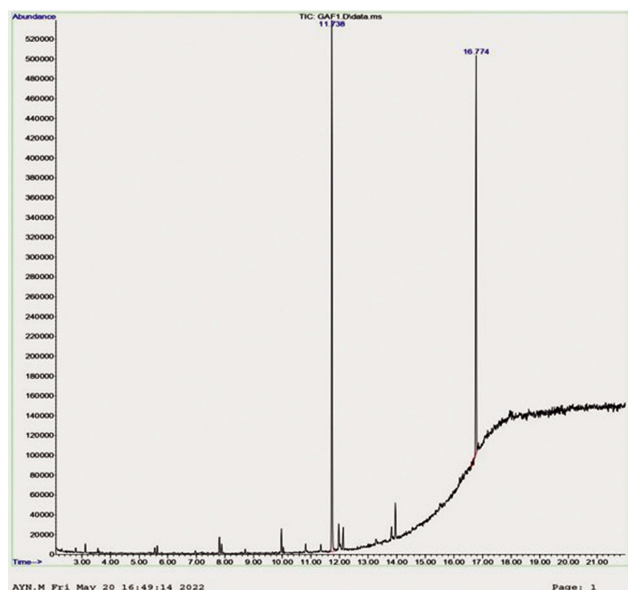

Fig. 3 GC- MS results of ethyl acetate extract of *Aspergillus flavus* confirms the presence of Dibutyl Phtalate and Pthalic acid, di (oct-3-yl) ester.

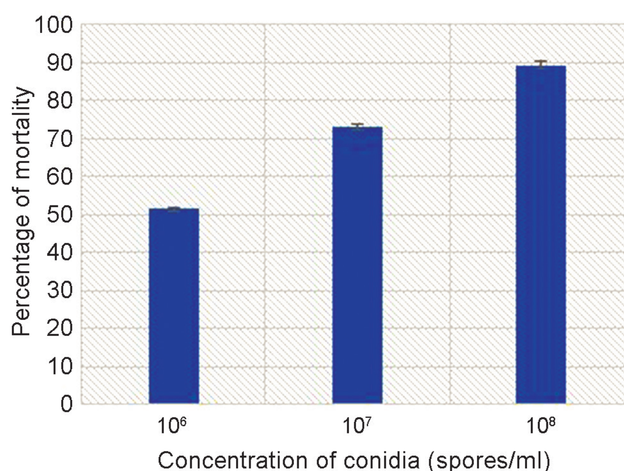

Fig. 4 The percent mortality of different conidial concentration (10^6, 10^7, 10^8) of *Aspergillus flavus* against *Phenacoccus solenopsis* after 5 days of treatment.

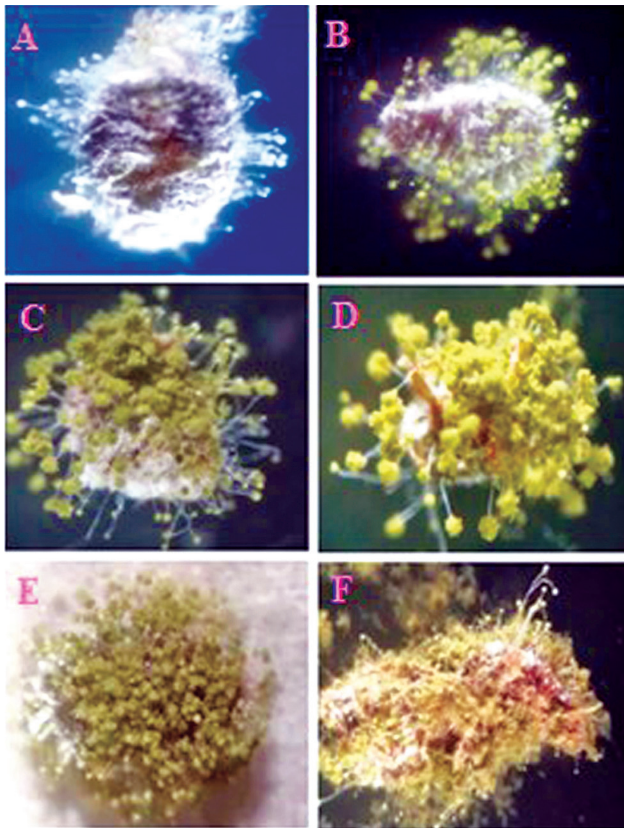

Fig. 5 Development of mycosis after exposure to *Aspergillus flavus* at conidial concentration of 10^8 conidia/mL (A–F) Stages of infection.

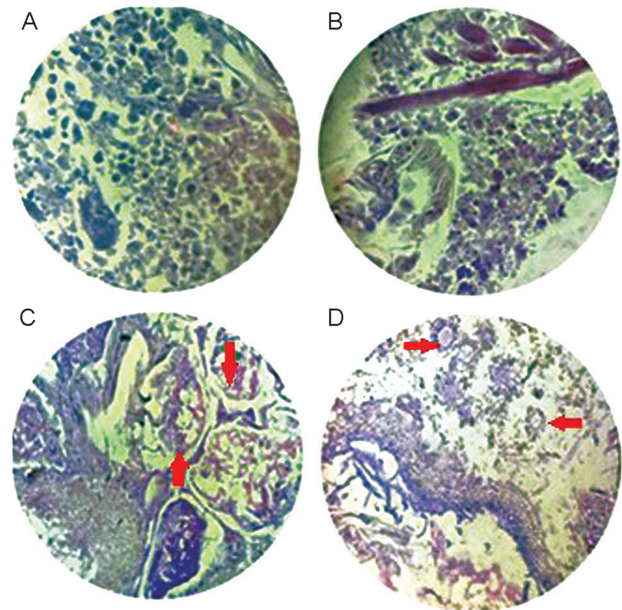

Fig. 6 (A), Micrographs of transverse section of *Phenacocccus solenopsis* stained with Hematoxylin-eosin; (B), Untreated insects without any cell damage (Control); (C), Insects treated with *Aspergillus flavus* conidial suspension (10^8 conidia/mL) results in destruction of tissues; (D), Spores were observed in the epithelial region as a result of mycosis.

A. flavus around the dead treated insects after 3 days of incubation. No mycelial growth was observed on dead control nymphs. This suggested that spores of *A. flavus* that adhere to the cuticle of infected nymph led to their death. The stages of mycosis were observed in dead infected insects (Fig. 5). Attachment of the fungal conidia onto the surface of insects is the initial stage of fungal infection. The ability of fungal spore to adhere and penetrate into the host cuticle determines its virulence and efficacy against insects. After the adhesion process, the spores undergo germination. The fungus starts to develop hyphae through the cuticle. During the infection process the fungal hyphae were spread over the host and secrete toxins. Then, the mycelium grows on the cadaver and produces new conidia. The persistence of spores on the infected nymphs is vital because when these fungal isolates are applied as mycopesticides, they must produce spores on the host cadavers which serve as a secondary source of culture for continuous propagation in the environment.

Histological studies were carried out to identify the cellular effect of the fungal infection in 3rd instar nymphs of *P. solenopsis*. The cross sections of the infected nymphs were observed through microscope at 40X magnification (LABOMED). Morphological alterations have been observed in the cuticle, tissues and mid gut. The cuticle and epithelial cells were destructed by the penetration of fungal conidia. The shape of cells and tissues of mid gut were deformed by the action of fungal metabolite. Spores of *A. flavus* were found around the epithelial region of the infected insects as a result of fungal infection. The histological studies revealed that the fungus caused morphological deformations in the treated nymphs by affecting their cells and tissues. The degree of adhesion and penetration of *A. flavus* to the cuticle surface of *P. solenopsis* nymphs was high, which showed the pathogenic effect of the fungal isolate (Fig. 6).

The results suggested that conidial suspension of *A. flavus* was most effective against the third nymphal instar of *P. solenopsis*. It is also revealed that *A. flavus* has the potential to infect the mealy bugs and its mycoinsecticidal properties were further substantiated by histological examinations. However, *A. flavus* itself cannot be directly employed as biocontrol agent due to its ability to produce aflatoxins. Consequently, identification and characterization of *A. flavus* derived secondary metabolites that are harmless to non-target organisms is crucial for harnessing their insecticidal properties and developing sustainable pest management strategies.

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