INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 14, No. 3, p. 133-139, 2019

RESEARCH PAPER OPEN ACCESS

PERSISTENCE OF LOCAL STRAIN OF ENTOMOPATHOGENIC FUNGI *ASPERGILLUS FLAVUS* IN INDOOR AIR

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Key words: *Aspergillus flavus*, Aflatoxins, Soil borne fungi, Conidia, Persistence, Indoor air.

http://dx.doi.org/10.12692/ijb/14.3.133-149 Article published on March 27, 2019

Abstract

To assess the persistence of entomopathogenic fungi *Aspergillus flavus* in indoor air, its local strain (FCBP-PTF-003) was isolated from Changa Manga forest near Lahore. *Aspergillus flavus* is considered pathogenic to insects but also have record of being dangerous to other organism and plants. Before using this fungus against insects, to assess its risk as potential human allergens, airborne conidia were measured in enclosed environments. To evaluate its persistence in indoor air, a spray formulation of *Aspergillus flavus* conidial solution was applied in a room selected for the experiment. Air sampling was carried out for three consecutive days with an interval of twenty four hours. Conidial count of CFU/m^3 obtained on media plates was recrded after five days of incubation. The conidial count of three consecutive days was recorded as 100,801, 57,940 and 3,184 CFU/m^3. Total viable airborne fungal counts varied as it reduced successively after three days. It was evident from the analysis that conidia of *Aspergillus flavus* do persist in air however the significant reduction in CFU/m^3 after time indicate that its persistence is not for the longer period of time. The local strain is found to be not much dangerous compared to the notorious reputation of *Aspergillus flavus*.

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Introduction

The entomopathogenic fungi are organisms that pose serious damage to insect species (Araujo and Hughes, 2016). These are often found in leaf litter and soil of world's forest ecosystem. It also occurs naturally as infections in insect hosts (Inglis *et al.*, 2001). Geographical location, climatic conditions, habitat type, cropping system, and soil properties have profound impact on the distribution and occurrence of entomopathogenic fungi (Meyling and Elinberg, 2006). The geographical attributes of regions of Punjab incalculably support the occurrence of entomopathogenic fungi of various genus and species including *Beauveria bassiana, Metarhizium anisopliae, Aspergillus flavus, Aspergillus oryzae, Fusarium oxysporum* etc. (Wakil *et al.*, 2014).

Aspergillus flavus is a filamentous fungus, which is known to reproduce rapidly in a vast range of environmental conditions. They can survive in temperature range of 12°C to 48°C, Whereas, there optimal growth temperature ranges from 28°C to 37°C, with humidity above 80% (Thathana *et al.*, 2017). Worldwide distribution of *Aspergillus flavus* is probably due to the production of airborne conidia, which is then dispersed by insects or air. *Aspergillus flavus* is mostly found to occur in the soil, where it, along with plant and animal debris, acts like an important nutrient recycler (Hedayati *et al.,* 2007). *Aspergillus flavus* along with other species of *Aspergillus* is regarded as opportunistic pathogen that requires weakened host or wounds for colonization. Although *Aspergillus flavus* has limited parasitic abilities it is associated with numerous diseases in plants, insects, man and other animals (Leger *et al.,* 2000). Isolates of *Aspergillus flavus* produce aflatoxins, these aflatoxins are notorious to have notable insecticidal, larvicidal and chemo sterilizing properties against a wide range of insect species (Drummond and Pinnock, 1990).

The aim of this study is to evaluate the persistence of *Aspergillus flavus* conidia in indoor air, to determine gradual difference of conidia over time and to determine relative percentage of persistence of conidia over time period of three days.

Materials and methods

The soil samples were collected from Changa Manga forest near Lahore. The soil samples were taken from 10 inches depth with the help of sterilized spatula and transported to laboratory in Environmental Science department of Lahore College for Women University, Lahore in properly labelled polythene bag. *Aspergillus flavus* was isolated by using the insect bait method (Zimmermann, 1986). The traditional bait insect used was the larvae of the wax moth (*Galleria mellonella)* (Chandler *et al.*, 1997). The pure cultures of required entomopathogenic fungi were obtained after mass culturing under controlled physical conditions (27°C to 40°C). Isolates of *Aspergillus flavus* were stored in cool temperature (4°C).

Microscopic, morphological and molecular identification

Slide culture was prepared to observe the microscopic characteristics of fungal isolate under compound microscope (Fig. 1).Morphological identification was done based on the shape of colony as well as the spores, the size of spores, Colony color, Margins, Colony reverse, Elevations, Nature of growth, and Nature of pores. The growth parameters like radial growth, spore count and days taken to cover up the full plate were also observed. The samples were also subjected to Scanning Electron Microscope for identification (Fig. 2). The DNA of isolated strain of *Aspergillus flavus* was extracted and sequencing was performed after quantification, PCR amplification, Gel electrophoresis and DNA Gel extraction.

Persistence of Aspergillus flavus in indoor air

To evaluate the persistence of conidia of *Aspergillus flavus* they were harvested by scraping the surface of seven days old culture gently with the help of sterilized inoculation needle. 0.1g *Aspergillus flavus* was added in a conical flask. 10ml of oil formulation was prepared (5ml Rosemary oil + 5ml Lemongrass oil) and added in conical flask containing 0.1g of fungi. The hyphal debris was removed by filtering the

mixture through fine mesh sieve. It was placed on bath sonicator for 3 minutes to break all the conidial fragments. The conidial concentration of final suspension was determined by direct count and concentration was adjusted to 10^8 using haemocytometer.

The solution was sprayed on a black cotton sheet of dimensions 1m×1m. Black cotton sheet was then upheld with thread secured to the both walls of the room. Air samples were collected with Sampl'air Lite into petri-dishes containing SDA. Air samples were taken before the experiment and after the experiment for three consecutive days with interval of 24 hours on SDA plates. Plates were sealed with white tape and stored in a Laminar flow for 5 days at room temperature. The presence of fungal growth in the plates was examined under microscope and morphological identification was performed. Direct conidial count of CFU/m^3 obtained on media plates was recorded after five days of incubation. The data was tabulated and showed in the form of graphs.

Results and discussion

The morphological and microscopic observations showed that the colonies were floccose, olive to dark green in color with white mycelium and reverse un-colored and became raised with time. Conidial head was radiated to columnar. Conidiophore was 400-800×8-16 µm in length; walls were rough, colorless to pale brown in color. Vesicle were spherical to elongate, 20-40µm wide, variable in serration, mostly uni-seriate, metulae were covering entire surface of vesicle and was 8-10×5-7µm. Phialides were 7-12×3-4µm. Conidia were globose to ellipsoidal, 3-6µm in dimeter with smooth to roughened walls (Table 1-2).

Table 1. Morphological Characteristics of *Aspergillus flavus*.

Morphological characteristics	
Colonial Features	*Aspergillus flavus*
Colony colour	Yellowish green
Margins	Entire
Colony reverse	Colorless to yellow
Elevations	Umbonate
Nature of growth	Rapid
Nature of pore	Powdery

Table 2. Microscopic Characteristics of *Aspergillus flavus*.

Microscopic Characteristics	
Hyphae	Branched septate
Conidiophore	Rounded
Length	400-800 × 8-16 µm
Diameter	20-25 µm
Vesicle	Spherical to elongate
Conidia	20-45 µm
Heads	Radiate to columnar
Diameter	3-6 µm
Ornamentation	Smooth to roughened
Color	green

The fungus was identified as *Aspergillus flavus* confirmed and was assigned accession number FCBP-PTF-0003 from fungal bank of Institute of Agricultural Sciences, University of the Punjab, Lahore (Fig. 3-5). For the sequencing the gene, sequencing was performed utilizing 18S rRNA gene of isolated Aspergillus flavus when subjected to BLAST search device in database of NCBI to distinguish the isolated strain.

According to the official website of allergens (www.allergen.org), several species of *Aspergillus,* including *A. fumigatus*, *A. niger*, *A. flavus*, and *A. oryzae*, are allergic. To date, 21 known and 25 predicted allergens of *A. fumigatus* have been identified. However, it must be noted that molds are not predominant allergens and that outdoor fungi are more important than the indoor ones (Singh *et al.,* 2014).

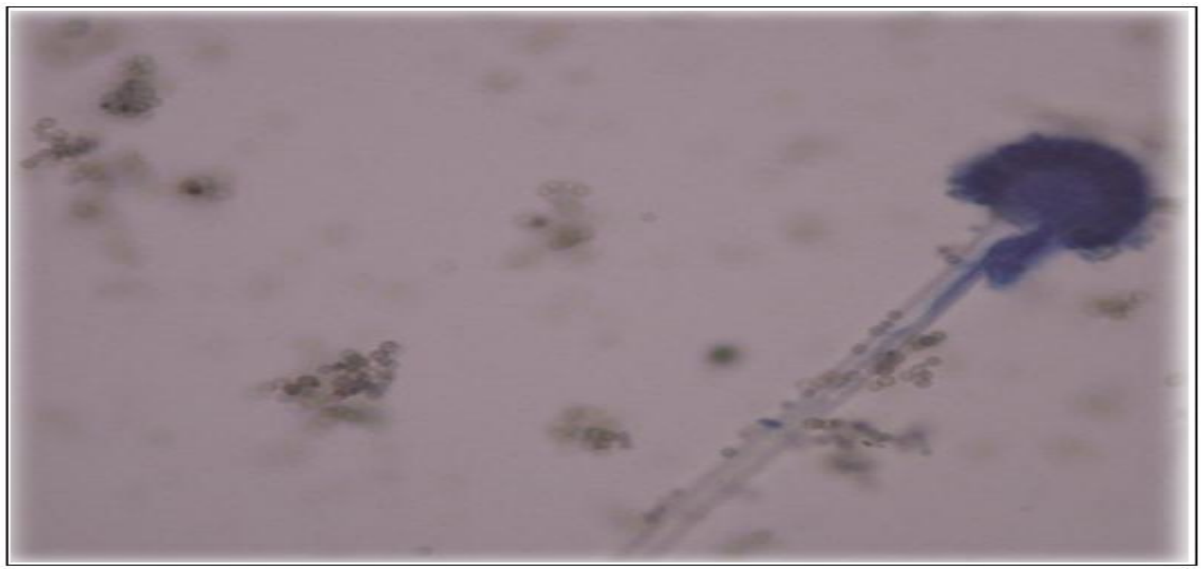

Fig. 1. Microscopic view of *Aspergillus flavus* via Compound Microscope.

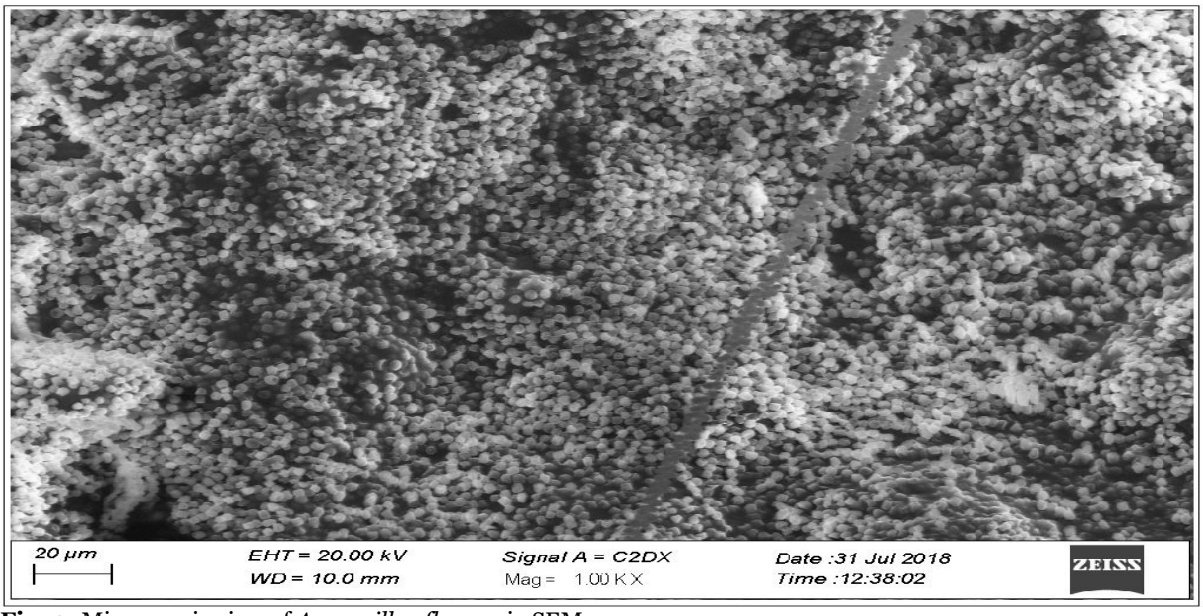

Fig. 2. Microscopic view of *Aspergillus flavus* via SEM.

In the present study a reduction in conidial persistence was seen over three days comprising of 100801 conidia per plate on the first day which declined to 57940 conidia per plate on 2nd day to 3184 conidia per plate on the 3rd day. Conidial counts were performed by haemocytometer (Fig. 6).

The conidia of *Aspergillus flavus* showed high persistence in indoor air immediately after the spray on the black cotton cloth.

The observed data of three consecutive days revealed that there was highest number of conidia on the first day post-application of spray which was reduced on second day and third day. It was concluded that conidia of the local strain of *Aspergillus flavus* did not persist for longer period of time and gradually reduce in number.

In a study conducted by Darbro and Thomas, 2009 the conidial viability of 10 entomopathogenic fungi was assessed. No isolate persisted longer than 1 week.In case of *Metarhizium anisopliae*, the Conidia were detectable immediately after treatment, with concentrations of 7000/m^3, decreasing over 500 conidia/m^3 after 48 hours.

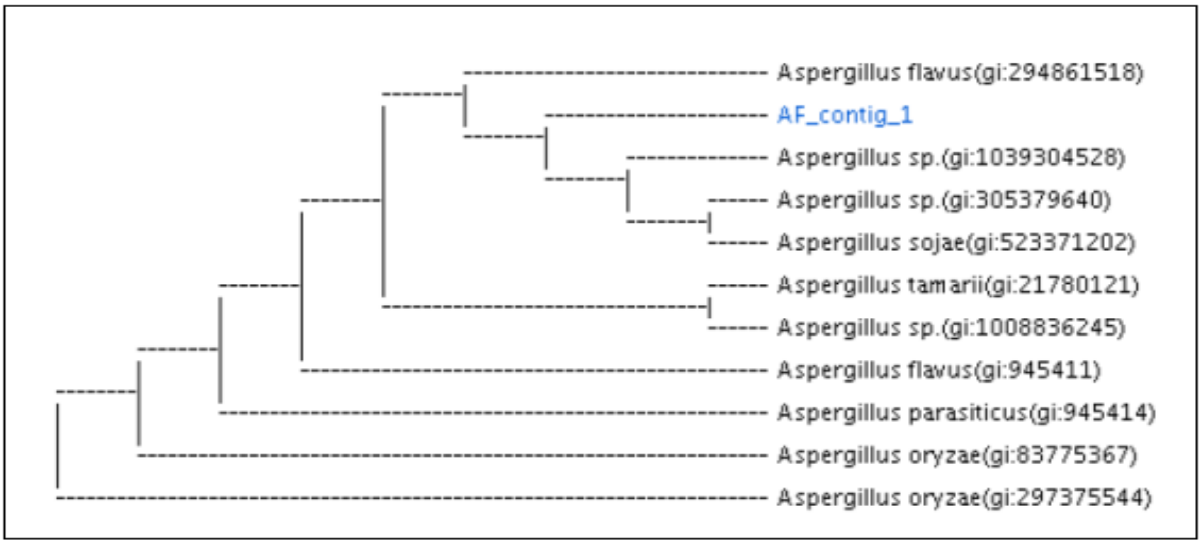

Fig. 3. Detail of the phylogeny containing the considered aspergillus sp.

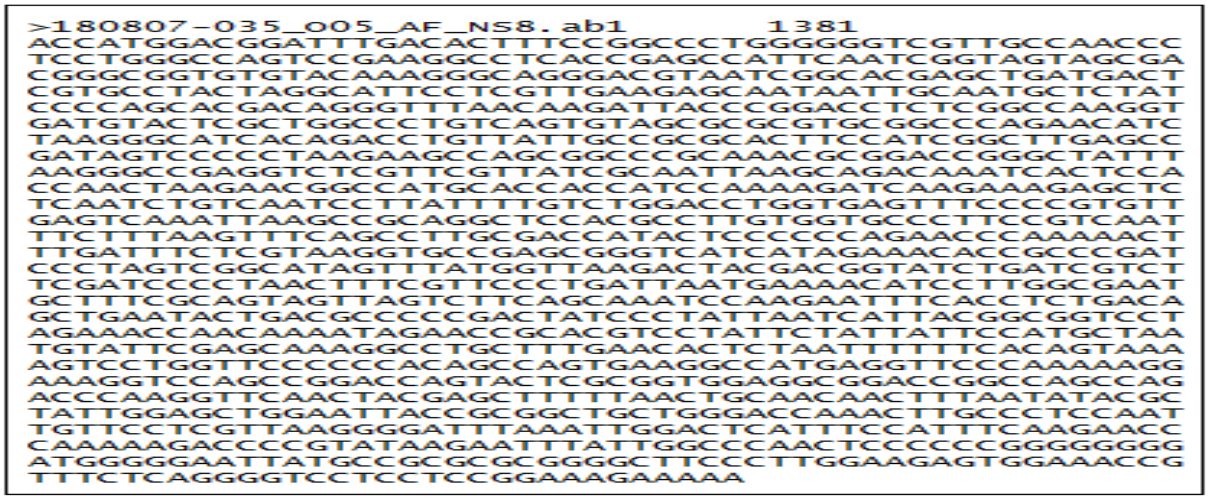

Fig. 4. NS1 5' (GTA GTC ATA TGC TTG TCT C) 3' Primer Sequence.

The present study showed highest concentration of conidia just after the oil formulation spray of *Aspergillus flavus*. Study by Alves *et al.*, (2002) revealed that oil formulations enhance the endurance of conidia against abiotic factors.

This can be the reason because of which spores remained stick to the black cotton cloth and showed high persistence after the spray. While after 24 hours the concentration of spores sharply declined and further reduced in number after 48 hours. The reason why the concentration of spores fell so drastically can be supported by the findings of Thomas *et al.*, 2005, who suggested that the drastic decline of spore concentration is an indicator that any number of spores in the air are not frequently freed, rather they rapidly dispose of.

They have argued that the use of fungal bio-pesticides do not add-in as much to the already present aero allergen burden of our local surroundings.

The prevalence of entomopathogenic fungi conidia in air is also dependent on the season and physical characteristics of the indoor environment (Rosas *et al.*, 1997).

A point by point inquiry is important to survey the introduction levels, impact of an atmosphere conditions and in addition the clinical parts of the nearness of unfavourably susceptible parasitic spores (Reddy and Srinivas, 2017).

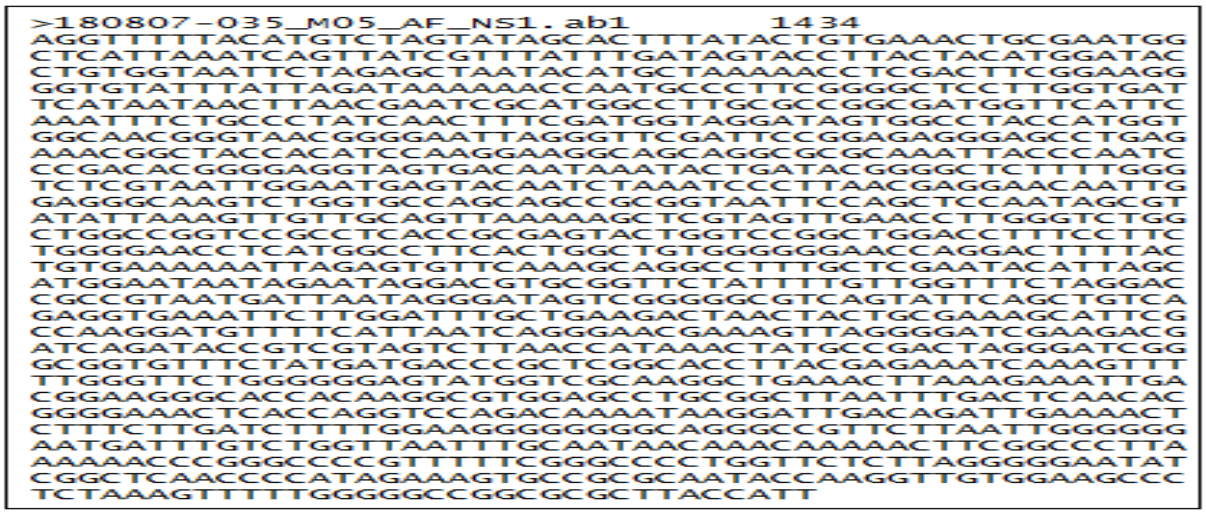

Fig. 5. NS8 5' (TCC GCA GGT TCA CCT ACG GA) 3' Primer Sequence.

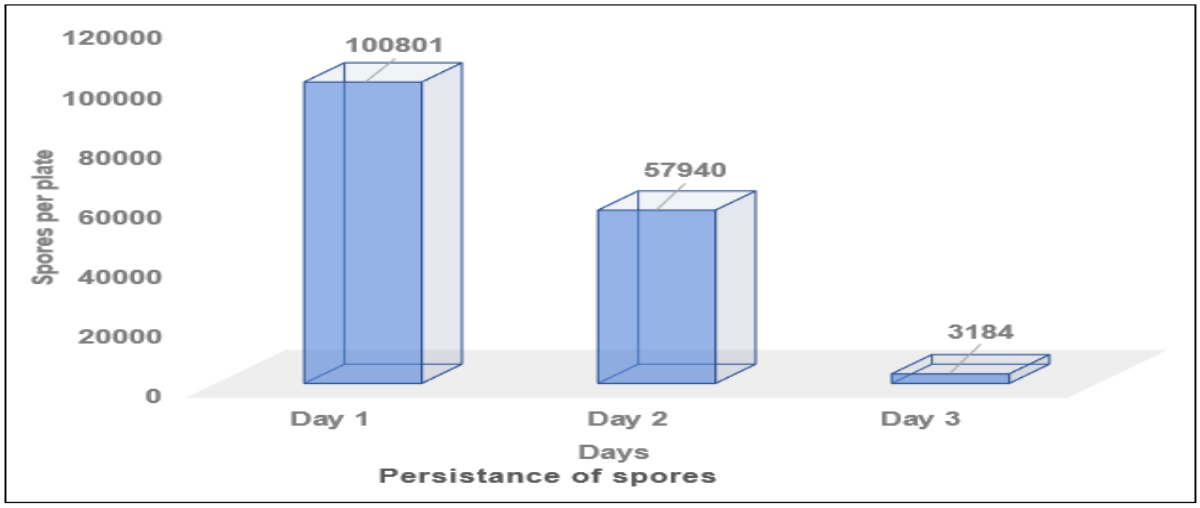

Fig. 6. Persistence of conidia of *Aspergillus flavus* in indoor air.

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