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Assessment of contamination levels and characterization of Aflatoxin B_1 producing *Aspergillus flavus* strain from food and feed samples from local markets of south India

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ABSTRACT

Aflatoxins are the toxic secondary metabolites produced by fungi *Aspergillus flavus and A. parasiticus* in many food and feed products including oil seeds when stored under ambient conditions. Aflatoxin B_1 a prototype of the aflatoxin is widely recognized as group I human hepato-carcinogenic compound by IARC. Contamination of oil seeds with the *Aspergillus Sps* and aflatoxin possess serious threat to the food and feed industry. The present study on the incidence of seed borne infection and evaluation of aflatoxin B_1 contamination levels in oil seeds by applying the principles of standard blotter method and chromatography methods respectively. The molecular characterization of toxigenic isolate No.39 by PCR method. The percentage incidence of seed borne infection was observed to be higher for the groundnut sample showing the mean percentage of infection to be about 48.57% than with all the seed samples. Among 55 isolates of *Aspergillus flavus*, 42 were found to be aflatoxigenic strains by TLC and amount of aflatoxin B_1 in oil seeds was found to be in the range of 29-35µg/ml by HPLC. The molecular characterization of toxigenic isolate was found to be closely related to *Aspergillus flavus*_strain DAOM 225949 and the nucleotide sequence was provided with a GenBank accession number JN938987. This study concluding that the oil seeds were prone to fungal infection and contamination with aflatoxin B_1 when stored under ambient conditions. So, proper storage, improvement of Good Agricultural Practices and regular inspection should be practiced ensuring that the safety of animals including human beings.

Key words: Food; Oil seeds; *Aspergillus flavus*; aflatoxin; TLC; HPLC; PCR; Phylogenetic analysis.

INTRODUCTION

Aflatoxins are a group of closely related heterocyclic compounds and toxic secondary metabolites produced predominantly by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Yu *et al.*,2002*)*. Of at least 16

structurally close aflatoxins has been identified, aflatoxins-B_1, B_2, G_1 and G_2 are most important toxins (Goldblatt et al,1969). Most studied aflatoxin is aflatoxin B_1 as it is recognized as most toxic metabolite and classified as group I human carcinogenic compound by the international agency for research on cancer (Bennet and Klich, 2003). Aflatoxin contaminates a vast array of food and agricultural commodities such as cereals, nuts, dried fruits, coffee, cocoa, spices oil seeds, dried peas and bean and fruit (Turner *et al*., 2009: Reddy *et al*. 2011). *Aspergillus* species can grow on a variety of substrates under different environmental conditions (Reddy *et al*. 2011). Oil seeds are the most widely distributed food crops in the world and its contamination by these seed borne fungi can lead to mycotoxin accumulation during the stages of growing, harvesting, storage, transporting and processing. Aflatoxin contamination of foods and feeds has gained global importance because of its deleterious effects on human as well as animal health (Okoli *et al*. 2006). Mycotoxins contamination intensity in food crops vary geographically and groundnut is main source of mycotoxins. Groundnut seed is predominantly infected with *Aspergillus flavus* and *Aspergillus niger* (Gebreselassie, Dereje, & Solomon,2014). The reported outbreak s of aflatoxicosis in man were due to the consumption of aflatoxin contaminated food and feed products (Reddy and Raghavender, 2007). The economic consequences of mycotoxin contamination are profound, as the crops contaminated with elevated levels of mycotoxin are often destroyed (Fakruddin *et al*. 2015). Feeding livestock and poultry with aflatoxin contaminated feeds can cause death and immune suppression as well as growth reduction. Low yields of animals and crops can also be occurred due to aflatoxin contamination (Phillips *et al.* 1996). The chromatography techniques viz., thin layer chromatography (TLC) and high-performance liquid chromatography(HPLC) were extensively used for mycotoxin analysis, although recently an increase in the use of high performance thin layer chromatography (HPTLC) has been recorded. The accuracy of TLC is less than that of HPTLC, but the results recorded using HPTLC are like HPLC and more consistent than enzyme linked immunosorbent assay (ELISA) data (Jaimez *et al.* 2000). This study was undertaken to investigate the incidence of seed borne infection and evaluation of aflatoxin B_1 contamination levels in the selected oil seed samples by applying the principles of standard blotter method and chromatography techniques viz., TLC&HPLC respectively and the molecular characterization of toxigenic *aspergillus flavus* isolate SNF-19 based on the principles of colony PCR and partial 18S rDNA sequencing.

METHODOLOGY

Chemicals & Reagents:

Sabouraud dextrose agar/broth (SDA/SDB), Yeast extract sucrose (YES) and analytical HPLC grade solvents and culture media were purchased from Hi-Media Mumbai (India). The standard AFB_1 was procured from Sigma-Aldrich (Steinheim, Germany) and thin layer chromatography (TLC, Silica gel 60) plates from Merck (Demhadt, Germany).

Sample collection:

All the oil seed samples viz., Groundnut (*Arachis hypogaea*), Castor (*Ricinus communi*), Mustard (*Brassica juncea*), Sesame (Husked and DE husked), Sunflower (*Helianthus annuus*), Guizotia (*Guizotia abyssinica*), Soybean (*Glycine max*), and Cotton (*Gossypium*) were collected randomly and aseptically following the standard microbiological protocol. The sampling sites comprises the market yards which are situated in major oil seed growing and selling regions of Karnataka, Tamilnadu and Andhra Pradesh(India). A total of eight distinct types of oil seed samples were collected and subjected to estimate the incidence of seed borne fungi and assessment of aflatoxin B_1 contamination levels in the oil seed samples.

Isolation and identification of seed borne fungi:

Standard blotter method.

The Standard blotter method was employed to evaluate the mean percentage of incidence of seed borne infection on contamination following the procedure of ISTA (1996). Seeds were surface sterilized with 0.1% mercuric chloride solution for 3-4 minutes rinsing thoroughly in sterile distilled water. About twenty-five seeds of each sample were placed in a petri dish containing three circular discs of moist blotting paper. The seeds were placed equidistant from one another and incubated at temperature of 26 ± 2^0 C for 7 days. The seed borne fungi were examined under microscope by staining with Lactophenol cotton blue (Fakhrunnisa *et al*. 2006). The *Aspergillus flavus* isolates among seed borne fungi were maintained on SDA slants as a pure culture for further research work.

Studies on effect of culture filtrates and analysis of AFB_1:

To study the efficiency of toxigenic and atoxigenic *Aspergillus flavus* isolates to produce aflatoxins and seed germination they were grown by inoculating the 1 ml of spore suspension into the sterilized fifty ml of yeast extract sucrose (YES) media and incubated under room temperature for 7 days. After the completion of incubation period, the culture fluid was filtered through Buchner funnel using Whatman's No.1 filter paper. The mycelia growth was discarded. The culture filtrates were used for the bioassay test and for estimation of aflatoxin B_1 following the protocol of Shukla *et. al*. (2008).

Studies on effect of culture filtrates on seed germination of fenugreek seeds

The toxicity of the aflatoxin obtained from culture filtrates of *Aspergillus flavus* on plant growth was assayed using seed germination and seedling vigour tests. Twenty ml of culture filtrates were utilized for the bioassay test. Fenugreek seeds were surface sterilized with 0.1% mercuric chloride solution for 3-4 minutes rinsing thoroughly in sterile distilled water before soaking in culture filtrates of various isolates of *A. flavus*. Seeds were given a 24-hour soaking in water and were plated equidistantly from one another on two moist blotters in horizontal and vertical rows.

A third blotting paper moistened with water was placed on the seeds. Then all the three blotting papers along with the seeds were rolled in and kept in a tray containing water. Care was taken not to immerse the seeds completely in water. Seeds soaked in sterilized water and in liquid media were kept as controls (Patil *et. al.* 2012). Each experiment was done in three replicates and control experiment was treated and maintained with distilled water in the same number of replicates. Results of seed germination were recorded after 7 days. Vigour index was also calculated using the following mathematical representation as described by Khokhar *et. al* (2013).

Vigour index = (Average root length + Average shoot length) x percentage of germination

Studies on analysis of AFB_1:

AflatoxinB1 (AFB_1) contamination levels in selected isolates were analysed by based on the chromatography methods by Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Aycicek, Hasan *et al*. 2005 & Younis M.H. *et al*.2003). The amount of AFB_1 was quantified by using following mathematical formula.

$$\text{Quantification of Aflatoxin } B_1 = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard weight}}{\text{sample weight}} \times \frac{\text{sample dilution}}{\text{standard dilution}} \times \text{purity} \times 100$$

Molecular characterization of toxigenic *aspergillus flavus* isolate SNF-19:

Genomic DNA isolation:

Based on the quantification of total AFB_1 from the selected isolates, the isolate SNF-19 was characterized molecularly based on 18S rDNA sequencing. Briefly, take 100 mg of fungal culture in a mortar and pestle. Add 750 µl of 1X suspension buffer crush the tissue, until it forms a fine paste and pipette into a 2ml vial. Add 250µl more of suspension buffer to rinse the mortar and to ensure that you are taking the entire ground tissue material into the vial. To this add 5µof the RNase A solution and mix 5-6 times by inverting the vial. Place it at 65ºC for 10 min with intermittent mixing. To the above add 1ml of lysis buffer provided. Mix 5-6 times. Keep the mixture at 65ºC for 15 min. Spin at 13000g for 1 min at RT. Collect the clear supernatant in a 2-ml vial. Load the supernatant on spin column (600µl each time) and spin at 13000g for 1 min at RT. Discard the contents of the collection tube. Place the spin column back in same collection tube. Add 500µl of 1X wash buffer to the column and spin at 13000g for 1 min at RT and repeat the previous step. Place the spin column in a fresh 1.5ml vial. Add 50 µl of warm elution buffer into the spin column. Keep the vial along with the spin column at 65ºC for 1 min. spin at 13000g for 1 min at RT.

Quantification of DNA with absorption:

The reliable amounts of DNA to fingerprint assays were obtained by further dilution of DNA concentration in TE buffer PH 7.6 at 1:7 (v/v) and measuring the absorbance at 260nm and 280nm wavelengths in a spectrophotometer the purity of DNA was checked by gel electrophoresis with 1% agarose in TBE buffer (Ausubel *et. al*., 1995)

PCR Amplification:

The genomic DNA of *A. flavus* isolate was PCR amplified using universal primers:

Forward primer aflr-F 5′- GTAGTCATATGCTTGTCTC-3' and Reverse primer afl- R 5'-GAAACCTTGTTACGACTT-3' corresponding to 18S rDNA.

Master mixture was prepared with PCR reagents and distributed into 200µl PCR tubes. The reaction volume of 50µl / reaction was maintained which comprised 1 µl of each primer. 20 pmol concentration 5 µl of 10 X PCR buffer, mixture of dNTP's each at a concentration of 200mM (1 µl) sterile double distilled water (40.75 µl), 2.5 U of taq polymerase (0.25 µl) and template DNA (1 µl). Reaction mixture without the Template DNA was maintained as negative control to check contamination. Amplification reaction was performed in thermal cycler (Eppendorf A. G. Barkhausenweg, Germany) for 35 cycles. The purity of the PCR product was checked by Electrophoresis with 1 % agarose following the procedure of Sambrook *et al*. (1989). The PCR product was sequenced by Sanger do deoxy method by genome biotechnologies, Pune. Nucleotide BLAST was performed to all ten obtained sequences in NCBI (www.ncbi.nim.nih.gov/) using blastn suite and top 5 hit sequences with more than 98% similarly to the query sequences were selected for further phylogenetic analysis. Multiple sequence alignments of all these sequences were performed by using CLUSTAL –X software version 2.1. Phylogenetic tree was constructed using the same software and the alignment data analyzed by neighbor – joining (NJ) methods. The sequences were deposited in NCBI Gen Bank with an accession number.

Statistical analysis:

Values were expressed as Mean ± standard error. Analysis of variance (ANOVA) was performed, and the differences between values were tested for significance by Turkeys multiple comparison tests employing the SPSS 20 (IBM, USA) programme. Differences at $p \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Oil seeds are more susceptible to fungal infection during storage, which are a reliable source for contamination with fungal toxins in the food stuffs. In the present study, a total of eight different oil seed samples were collected from major oil seed growing and selling regions of Karnataka and Andhra Pradesh(India) and subjected to estimate the incidence of seed borne fungi and assessment of aflatoxin B_1 contamination levels in the oil seed samples and the obtained results of seed borne fungi were presented in Fig 1.0 (A, B, C &D).

Seed borne mycoflora as observed by the standard blotter method.

The storage fungi like *Aspergillus flavus, A. niger, A. oryzae, A. flaviceps* dominated the mycoflora of groundnut and were recorded in all the samples. Apart from the *Aspergillus* species, *Penicillium* species were also recorded in almost all the samples. *Rhizopus* species and *Mucor* were also commonly encountered. In addition to the storage fungi, field fungi like *Fusarium* species were also observed.

In soyabean the incidence of *A. niger and A. flavus* was more as compared with the other *Aspergillus* species. *Aspergillus oryzae* was always frequent but its frequency was less compared to *A. flavus* or even *A. niger., Penicillium and Rhizopus* species were also frequently recorded. *Fusarium* species was found to be prevalent in three samples. *Trichoderma and Neurospora* species were also found, in three and one samples respectively.

The Mycoflora of castor includes the *Aspergillus* species*, A. flavus, A. versicolor and A. niger* were most frequently observed; but the frequency of *A. niger and A*. *versicolor* was less than *A.flavus. Phoma and Candida* species were also encountered. The incidence of *Penicillium* species was less while *Rhizopus* species were observed frequently. Among the field fungi *Fusarium and Curvularia* species were encountered. *Trichoderma* and *Neurospora* species were also recorded in many samples, but in lower frequencies.

As noticed in the above two cases, *Aspergillus flavus* was observed in all the samples of Husked (sesame white). *A. niger* was also noticed but the frequency was less. Among the field fungi, *Fusarium* species was the most common. *Curvularia* and *Trichoderma* species were also recorded

Among the *Aspergillus* species, *A. flavus* was most the most frequent in all the samples of sesame (Dehusked). *Aspergillus niger* were also commonly noticed but the frequency is less. Incidence of *Penicillium* species was comparatively less. *Mucor, Trichoderma* and *Fusarium* species were also encountered.

In all the cotton samples, *Aspergillus flavus* and *Aspergillus niger* were observed in alarming proportions. *Rhizopus, Fusarium, Trichoderma* species were also encountered in all the samples. *Chaetomium* species was also noticed in few samples.

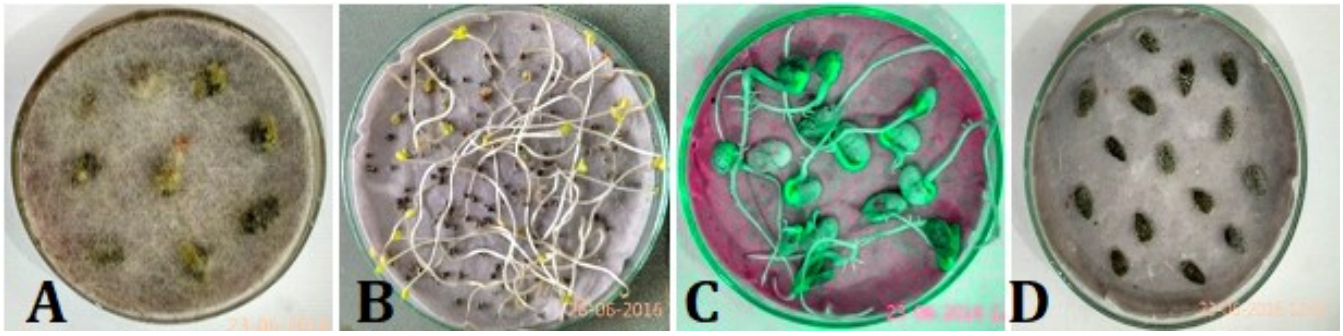


Fig 1.0 A. Seed borne fungi of a) ground nut b) sesame c) soya bean and d) sunflower oil seeds

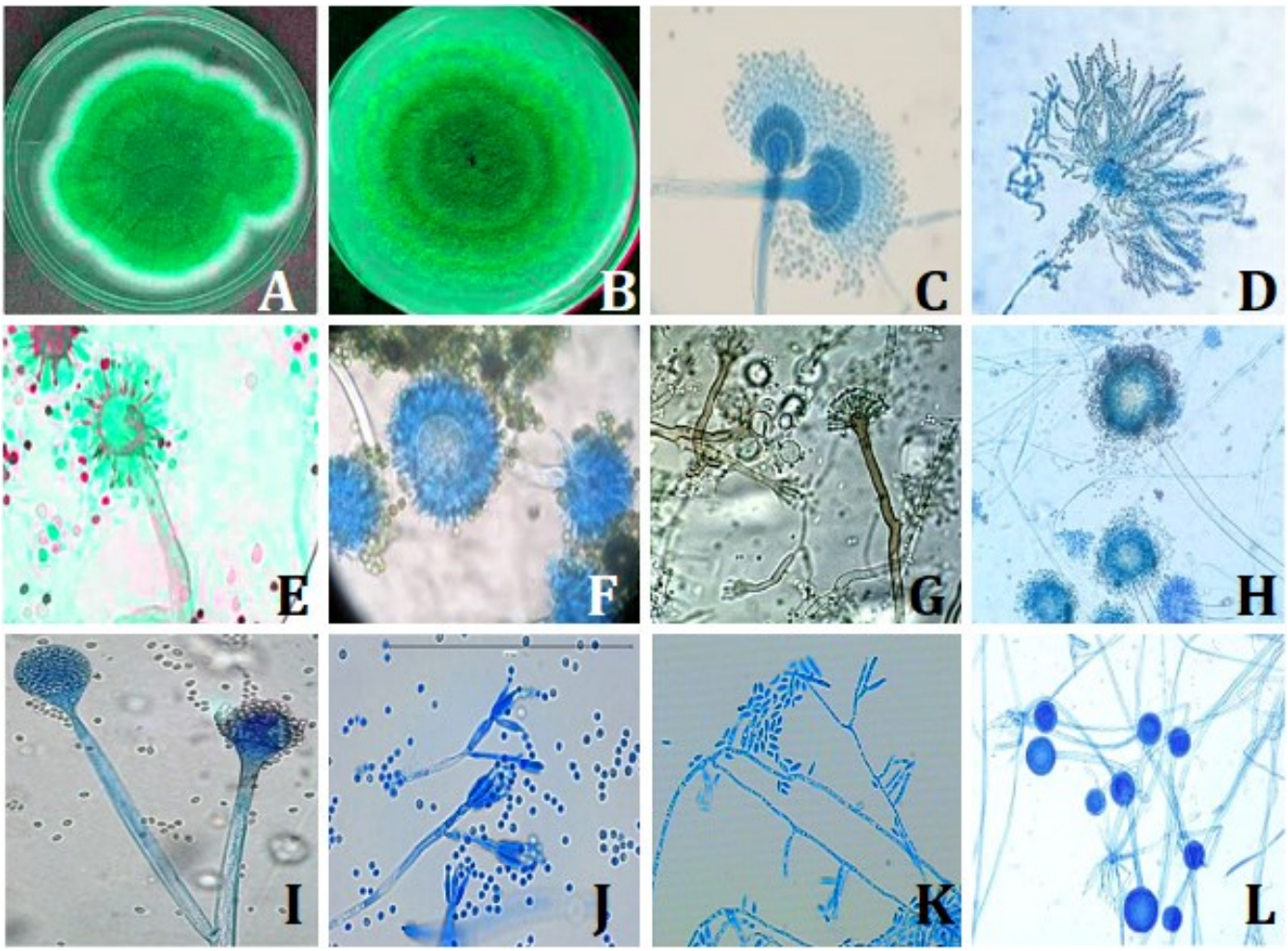


Fig 1.0 B. Pure culture: of a) *Aspergillus flavus* b) *Aspergillus fumigatus.* and **Microscopic view of:** c) *Aspergillus flavus* d) Microscopic view of *Aspergillus fumigatus.* e) *Aspergillus oryzae* f) *Aspergillus versicolar* g) *Aspergillus nidulans* and h) *Aspergillus niger*. i) *Mucor sp.* j) *Penicillium sp.* k) *Fusarium sp.* and l) *Rhizopus sp.*

In all the sunflower samples, *Aspergillus flavus* was encountered in alarming proportions. Incidence of *Penicillium* species was less when compared to *Rhizopus* species. In addition to these, *Candida, Diplodia, Neurospora, Macrophomina* and *Chaetomium* species were also encountered in less frequencies. Among the *Aspergillus* species of Guizotia samples, *A. flavus* was mostly noticed. *Rhizopus, Colletrotrichum, Mucor, Chetomium* and *Curvularia* species were also encountered in less frequencies.

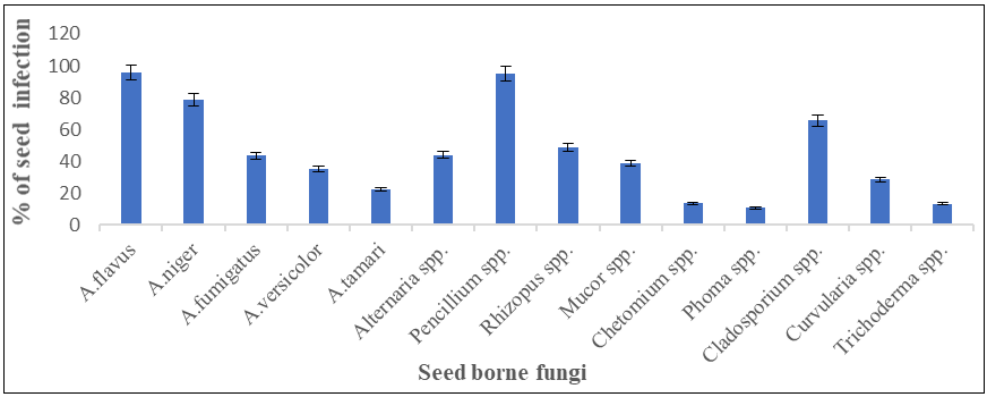
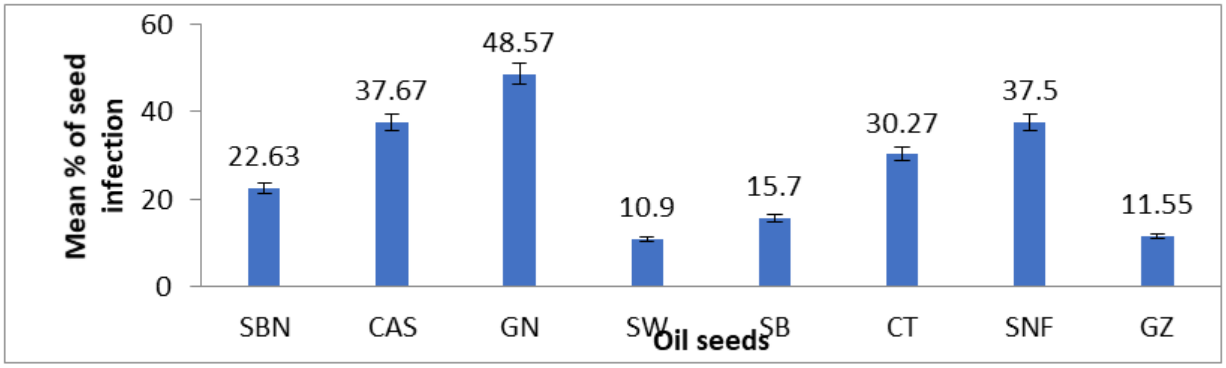
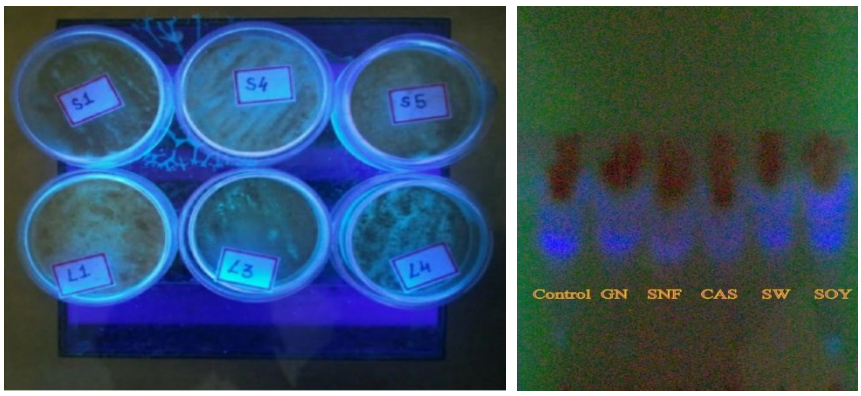


Figure 1.0 C. Percentage of seed borne fungi in oil seeds
Data given are mean of three replicates ±SE; anlysis of variance (ANOVA) $p < 0.001$



GN=Groundnut , CA=Castor , SNF=Sunflower, SBN=Soyabean, GT=Guizotia SB=Sesame black, SW=Sesame white, CT=Cotton

Figure 1.0 D. Showing the incidence of seed-borne mycoflora
Data given are mean of three replicates ±SE; anlysis of variance (ANOVA) $p < 0.001$



A) B)

Fig 1.1 A) SDA plates with *Aspergillus flavus* isolates observed under UV light (365 nm) **B)** TLC plate showing presence of AFB_1 of selected isolates with standard AFB_1 (control) under UV light (365 nm).

Studies on analysis OF AFB_1

Among 55 isolates, 42 isolates of *Aspergillus flavus* strains are found to be Aflatoxigenic in their nature based on the ability of AFB_1 production by qualitative method of TLC and the results of seed germination and vigour index, indicating that culture filtrates of different isolates were showed non-phytotoxicity by enhancing the growth of seedlings. From the quantitative method of HPLC, the amount of AFB_1 from selected isolates of seed samples was estimated and found to be in the range of 29-35 µg/ml in all the selected seed samples. (Fig 1.1, 1.2 and 1.3).

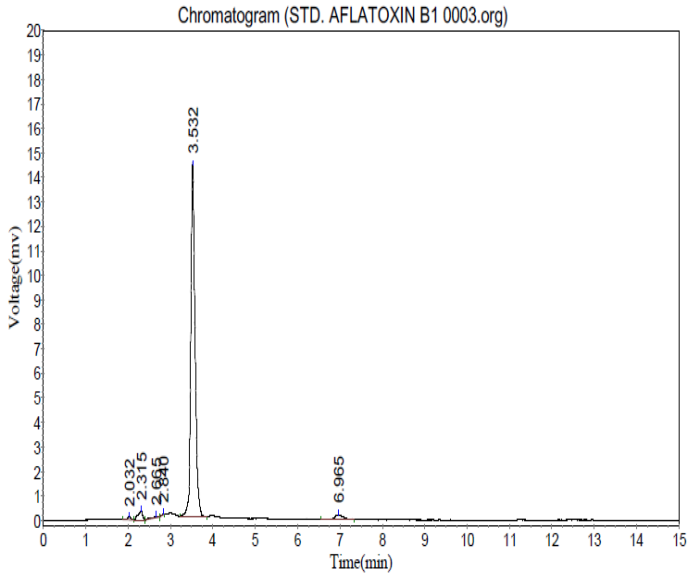


Fig1.2 Chromatogram of Standard AFB_1

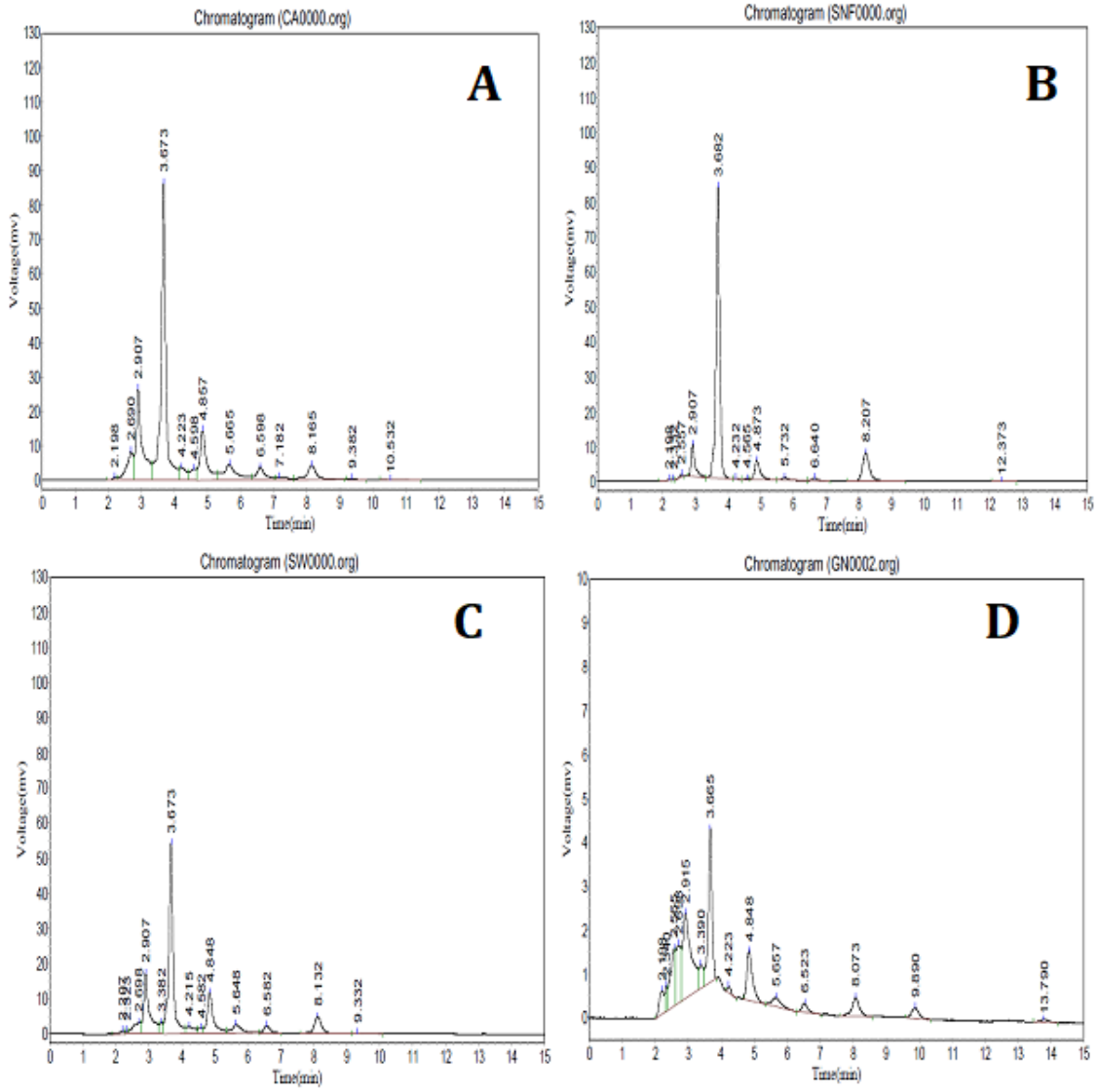


Fig1.3 Chromatogram of oil seed samples a) Castor b) Sunflower c) Sesame d) Groundnut.

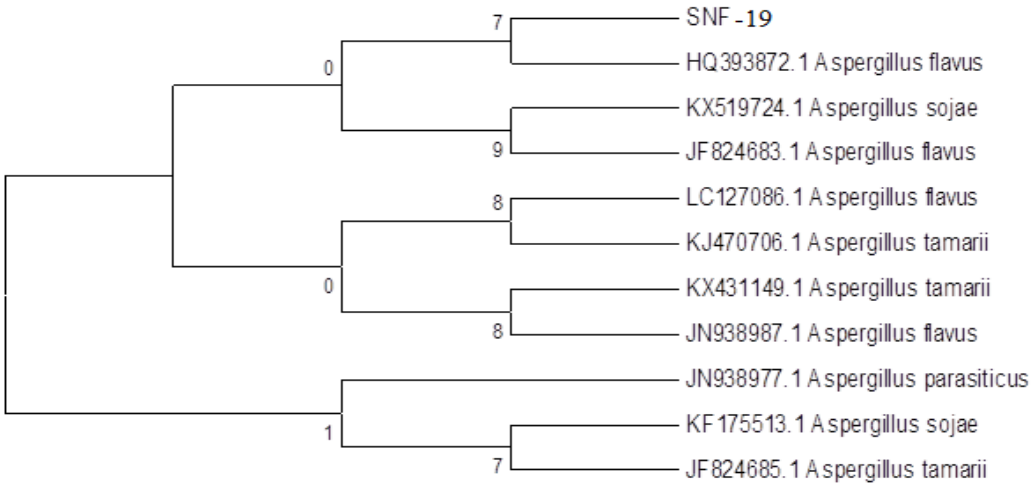


Figure1.4: Molecular Phylogenetic analysis by Maximum Likelihood method

Molecular characterization of isolate SNF-19:

The desired sub cultured organism isolate No. SNF-19 was subjected to genomic DNA isolation and sequencing by colony PCR method & analysed ABI 3500 XL Genetic Analyzer and the genomic DNA size has to be estimated 1.5 Kbp and the BLAST results were analyzed using NCBI database & distance tree was constructed using neighbor joining method.The BLAST result of selected aflatoxigenic fungal isolate SNF-19 was found to be closely related to *Aspergillus flavus* strain DAOM 225949 with 99% sequence similarity. The 760bp 18S rDNA nucleotide sequence was provided with a Gen Bank accession number JN938987 (Fig 1.4).

CONCLUSION

The present study investigates on the screening of seed borne mycoflora of various oil seeds by using standard blotter method, followed by identification of seed borne mycoflora using macroscopic, microscopic and molecular based methods like morphological, lactophenol cotton blue staining and PCR based method respectively. The percentage incidence of infection was observed to be higher for the groundnut seed sample showing the mean percentage of infection to be about 48.57% than with all the seed samples. The genus *Aspergillus* was most predominant, with *A. niger* topping the list followed by *Rhizopus spp*. and the least was *Trichoderma*. In addition to storage fungi, the field fungi *Fusarium* and *curvularia species* were recorded. The bioassay of culture filtrates of various *A. flavus* isolates showed non-phytotoxicity by enhancing the growth of seedlings. Among 55 isolates of *Aspergillus flavus*,42 were found to be aflatoxigenic strains by determinative method of TLC and amount of aflatoxin B_1 in oil seeds is estimated to be in the range of 29-35µg/ml by quantitative analysis of HPLC. The molecular characterization of toxigenic *aspergillus flavus* isolate was found to be closely related to *Aspergillus flavus* strain DAOM 225949 with 99% sequence similarity and the 760bp 18S rRNA nucleotide sequence was provided with a GenBank accession number JN938987. This study concluding that the oil seeds are prone to fungal contamination and production of aflatoxin B_1 when stored under ambient conditions. Therefore, proper storage, improvement of Good Agricultural Practices and regular inspection of its quality should be practiced ensuring that the safety of animals including human beings.

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