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IDENTIFICATION OF *ASPERGILLUS* SPECIES ISOLATED FROM CORN AND PEANUTS IN STORAGE GODOWNS

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ABSTRACT

The aim of this study was to identify aflatoxin producing fungi from affected corn and peanut samples collected from the storage godowns. Fungi were isolated from corn and peanuts using potato dextrose agar and modified rose bengal agar. The pure culture of the isolates were grown on differential media namely malt extract agar, czapek yeast extract agar and czapek dox agar for species identification using macro morphological characteristics. Lacto phenol cotton blue stains were used to identify fungal microscopic structures. All the fungi belonged to *Aspergillus species*. The fungal isolates were tested for aflatoxin producing ability. Out of the five different *Aspergillus species* isolated, two species namely *A.flavus* and *A.parasiticus* producedaflatoxin in the *Aspergillus flavus parasiticus* agar medium (AFPA) *and Aspergillus* differentiation medium (ADM).Early detection may help to bring in early management and control practices.

KEYWORDS:*Aspergillus*, **corn, peanuts, aflatoxin, mycotoxins.**

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INTRODUCTION

Aspergillus is a ubiquitous filamentous fungus that is both a saprophyte and an opportunistic pathogen of plants and animals.[1] *Aspergillus* species are used in the fermentation industry, but they are also responsible for various plant rots and food secondary rot, with the consequence of possible accumulation of mycotoxins. Though *Aspergillus flavus* has the ability to infect humans, its most serious threat to human health is through production of aflatoxin that contaminates food crops, including maize and peanuts.[2,3,4] The aflatoxin producing *Aspergillus flavus, Aspergillus parasiticus* and ochratoxinogenic *Aspergillus niger*, *Aspergillus ochraceus* species are frequently encountered in agriculture products.[5,6] *Aspergillus* can grow under conditions with very less moisture content. They are main contaminants in storage godowns affecting the quality of food and feed samples by altering the taste, reducing the nutrient levels apart from producing mycotoxins. Mycotoxins are carcinogenic to humans and animals as well as possess many phytotoxic properties.[7] The *Aspergillus* contamination of plant products in storage godowns brings in huge economic losses and health drawbacks to the farmers, food industrialists and consumers. Their early detection can help to take preventive measures to combat economic and health losses. Aflatoxin produced by fungal growth in food and feeds cannot be detected easily and rapidly without the help of specific tests involving special instruments which are costly and aflatoxin detection tests are not routinely adopted everywhere required. Eventhough traditional precautionary measures are present to keep in check the fungal contamination and growth, they are not actively practiced until any fungal infestation or infections are detected. Early growth detection of fungi should help prevent further growth and spread in the feed material especially during storage process in godowns. Deleterious fungal growth and aflatoxin detections in feed may warn the incharge personals and facilitate them to adopt the precautionary measures though it is not always possible to control their multiplication and spread completely. *Aspergillus* has the spore bearing structure called conidia head. The character that gives most trouble in *Aspergillus* identification is the presence or absence of metulae, a layer of cells between the vesicles and the spore forming phialides. The pear shaped phialides are either unisertiate or bisertiate conidial heads. For their identification macro and micro morphological characteristics are seen on differential growth media. The macro morphological features included colony colour and texture, presence of exudates, and production of soluble pigments by the fungi in the media with formations of sclerotia, and reverse colour of the plate. The micro morphological characteristics include shape of conidia head, seriation, vesicle shape and diameter, stipe - length, width, texture and colour, conidia size, shape, texture and colour; size of sclerotia which are noticed under microscope. Morphological methods have been supplemented by molecular and immunological approaches.India is a tropical country with warm humid climate.The study area Namakkal, located in Tamilnadu state of south India bears a tropical climate and is bestowed with many peanut and corn processing units where fungal infestations are frequent problems and economic losses due to mycotoxins producing fungal growth are encountered continuously.Continuous monitoring and checking the quality of the stored crops are important which are intended to be processed for food or feed preparation.The main objective of this study was to isolate the prevailing fungal types in post-harvest food crops stored in godowns and their ability to produce aflatoxin is assayed.[8]

MATERIALS AND METHODS

Sample collection

The study was carried out in selected storage godown which have shown high infestation and crop damage with huge economic loss during the previous season. From the peanut storage godown located adjacent to the three peanut processing oil mill units in Namakkal district, five kgs of peanut samples were pooled by following random sampling technique, collecting half a kg from 10 different sites in the storage godown. From the whole pooled lot, the fungal infected kernels were separated out aseptically. Their weight wasmeasured using a digital balance. The same sampling procedure was followed for collecting infected corn kernels from a corn storage godown located in a village adjacent to Namakkal district. The percentage of infected nuts and kernels present in the collected samples were calculated based on the counts and weight.

Isolation and identification of moulds[9]

For fungal culturing direct plate inoculation technique was used using 50 infected nuts and kernels obtained.Using 95% ethanol-flamed forceps placedthe individual nuts or kernels on the surface of solidified agar.The corn kernels and peanut seeds were directly inoculated into PDA plates and incubated for 5 – 7 days at room-temperature along with a negative control plate with no inoculation. The observed colonies were sub-cultured onto Rose-Bengal chloramphenicol media. The colony morphology and appearance of *Aspergillus*strains differ based on the media used. Hencepure cultures of *Aspergillus* species were grown on three fungal differential media - Malt Extract Agar (MEA), Czapek Extract Yeast Agar (CYA) and Czapek Dox Agar (CZ) and incubated at 25˚C for seven days. Colony morphology and appearance of isolates were observed both in the top side and bottom side of the media. Micro morphological characteristics of the pure culture colonies like conidiawere observed as wet mount in Lactophenol cotton blue stain for identification to species level by the conidiospore appearance and arrangement.

Aflatoxin production[10]

In the present study, *Aspergillus flavus parasiticus* agar medium (AFPA) and *Aspergillus* differentiation medium (ADM) media were used to detect aflatoxin producing ability of the fungal isolates. A bright orange colour on the reverse side of the plates of *Aspergillus flavus parasiticus* agar medium (AFPA) and *Aspergillus* differentiation medium (ADM) will indicate a positive result.

RESULTS

Fungal Infection percentage

Out of the collected 5 kg of ground nut when the infected nuts were separated and weighed they showed a 40gm of weight giving an infection weight percentage of 8 and an infection count percentage of 15. From the collected 5 kg of corn kernel sample, the infected kernels were separated and their weight was measured to be 30gm giving an infection weight percentage of 6 and an infection count percentage of 13.

Fungal isolation in basal media

The colour of colony was used as the first level of identification. From the observations made in infected peanut and corn culture plates the fungal growth was seen in all the inoculated plates. In peanut inoculated plates 45 different non overlapping fungal colonies were picked and followed with pure culture technique. Among them 28 colonies were in greenish shade. Other10black colonies, 5 brown shaded colonies and 2 grey shaded colonies were obtained.The observation of plates of the infected corn kernels revealed 35 non overlapping colonies which were also subjected to pure culture techniques. From the wet mount preparation using 10 % KOH *Penicillium* and *Rhizopus sp.* were identified along with *Aspergillus sp.* All the isolates belonging to genus *Aspergillus* were selected for the study. *Aspergillus niger* species were identified by their colonies in shades of black and brown. The colonies of *Aspergillus flavus and Aspergillus parasiticus* were either yellow green, green or deep green in colour. Other features included production of exudates and soluble pigments in the media. Based on morphological similarity of the colonies in the peanut sample, the number of *Aspergillus niger, Aspergillus neoniger, Aspergillus violaceofuscus and Aspergillus flavus* obtained were 6, 6, 2 and 26, respectively. In the corn sample all the four types of fungi similar to peanut sample were obtained with *Aspergillus parasiticus* in addition and the number of colonies obtained were 6,4,2,21 and 2 respectively.

Aflatoxin producers

Isolates belonging to *Aspergillus flavus or Aspergillus parasiticus* exhibited a bright orange colour on the reverse side of the plates of *Aspergillus flavus parasiticus* agar medium (AFPA) and *Aspergillus* differentiation medium (ADM). This confirmed the aflatoxin producing ability of the *Aspergillus flavus* or *Aspergillus parasiticus* isolated from corn and peanut samples. In the present study the percentage of aflatoxin producing species were equally distributed between the corn and peanut samples.

Morphological Characteristics and Fungal identification

Aspergillus parasiticus

The colonies were olive green on MEA; they had white mycelia and formed furrows in the colonies; produced brown sclerotia, exudates but no soluble pigments. Reverse was light brown with radiating furrows (Fig 1- a & a1). On PDA the colonies were yellow green with white to cream mycelia and yellow green edges. Sporulation rings formed in the colonies. The colonies produced exudates and soluble pigments with cream to pale yellow colour on the reverse (Fig 1- b & b1); On CYA the colonies were yolk yellow to green conidia with white mycelia and velvet texture. They produced small conidia heads, sclerotia and exudates present, reverse colour was straw (Fig 1- c & c1). Yellow colonies with white edges of young mycelia were formed on CZ with very slow growth and formed furrows. They produced exudates and brown sclerotia but no soluble pigments. Mycelia were cream to white and formed a mat beneath the colonies (Fig 1- d & d1). They produced small conidia heads, sclerotia and the hyphal threads were very conspicuous.[10] In this study it was observed that the scattered colonies which were whitish mycelium initially changed to light green colour spored colonies in Rose Bengal agar unlike in PDA were the profuse growth with green colouration appeared even earlier.

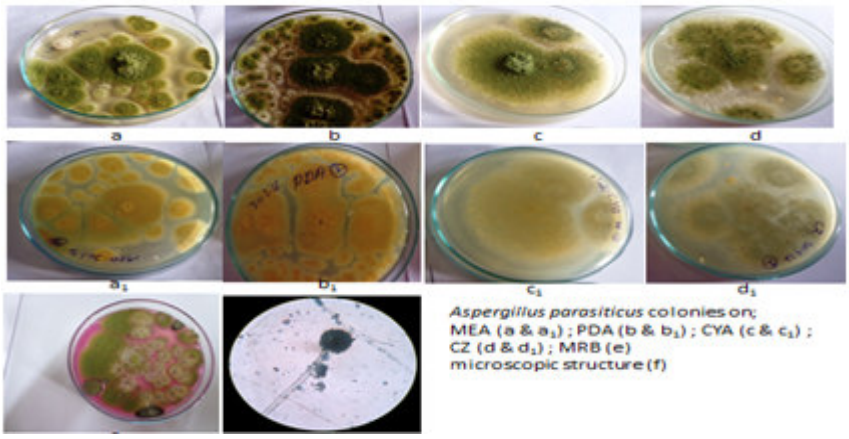

Figure 1
***Aspergillus parasiticus* colonies and microscopic structure**

Aspergillus niger

Colonies after 7 days of incubation at 25˚C on MEA colonies were date brown with white to cream thick mat of floccose mycelia beneath the colonies and at the edges. It formed radial furrows very close to each other. They lacked soluble pigments and exudates. Reverse was brown; on PDA agar the colonies were similar to that of MEA but much larger in size; Colonies on CYA were white to olivaceous in colour with a thick mat of white mycelia beneath the colonies formed radiating

furrows on the colony. There was fast growth of the colonies which produced exudates but no soluble pigments. Reverse was pale brown to yellow. On CZ the colonies were olivaceous buff changed colour to brown with age. They produced black conidia at the centre and white mycelia towards the edge. Exudates were present. Reverse was greyish to straw.

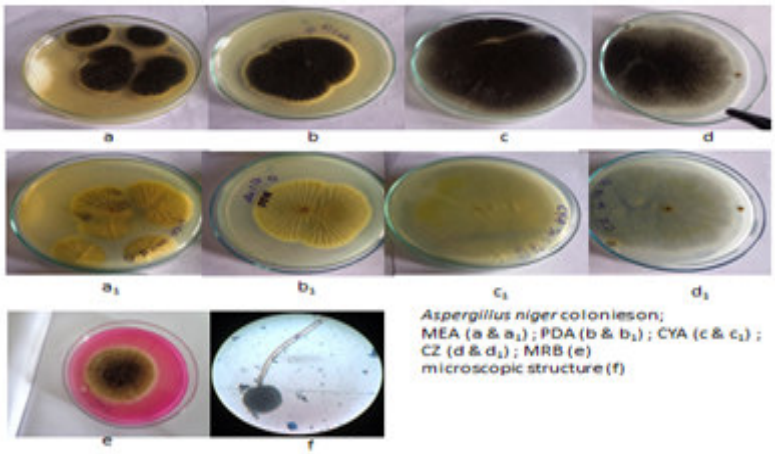

Figure 2
***Aspergillus niger* colonies and microscopic structure**

Aspergillus neoniger
The morphological appearance was comparatively similar with the results of *Varga et al.,*[6] in MEA and CYA medium (Fig-3) and hence suggested to belong to the species *Aspergillus neoniger.* Also the other features which confirmed the species are - Conidiophores biseriate with globose vesicles, stipe smooth-walled to finely roughened brown, coarsely roughened to echinulate. Sclerotia not observed.

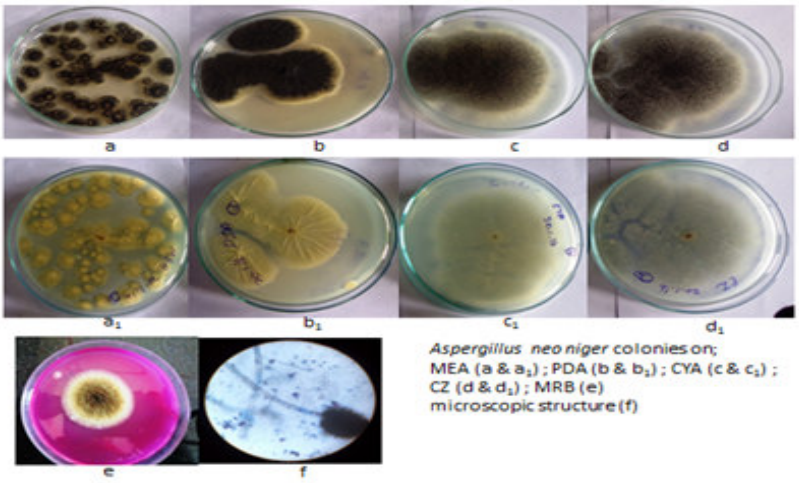

Figure 3
***Aspergillus neoniger* colonies and microscopic structure**

Aspergillus violaceofuscus
Colony reverse colour on CYA showed brown to cream yellow. Conidiophores were unisertiate with globose vesicles; smooth-walled to finely roughened stipe and hyaline in nature. Conidia were ellipsoidal to slightly fusiform, brown, and coarsely roughened to echinulate. Sclerotia not observed. The colonies were similar to *A. aculeatus* in colony and microscopical appearance while comparing the study results of Nyongesa *et al.*, 2015 but the colony appearance and descriptions matched much with that of the results of Varga *et al.,* 2011 and hence reported to be *A. violaceofuscus*.[10,11] Raper & Fennell considered *A. violaceofuscus* as a possible synonym of *A. aculeatus*.[12]

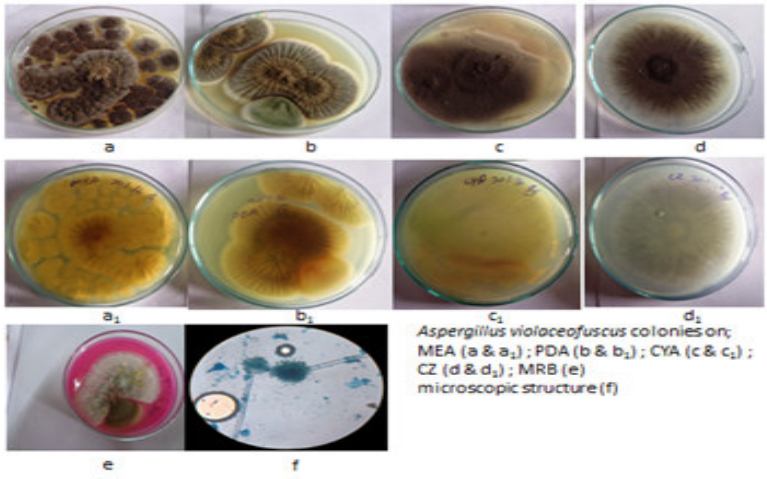

Figure 4
Aspergillus violaceofuscus colonies and microscopic structure

Aspergillus flavus

Colony after 7 days of incubation at 25˚C on MEA were yellow green with white mycelia at the edges; formed sporulation rings; the conidia were rough; did not produce exudates and soluble pigments; Reverse colour was cinnamon brown. Greenish colonies appeared on PDA medium; Colonies on CYA were yellow at the centre with white mycelia at the edge; conidia were rough; lacked exudates and soluble pigments. Reverse colour was straw. The colonies on CZ were white to yellow with scattered conidia, had fluccose texture; no exudates and soluble pigments. Reverse colour was yellow to cream. Predominantly the species were unisertiate but some were biseriate; conidia heads were radiate to columnar with loosely packed phialides; theunisertiate conidia heads had radiate vesicle with the philiades covering upto three quarter of the vesicle; while bisertiate the vesicles were spherical to globose.

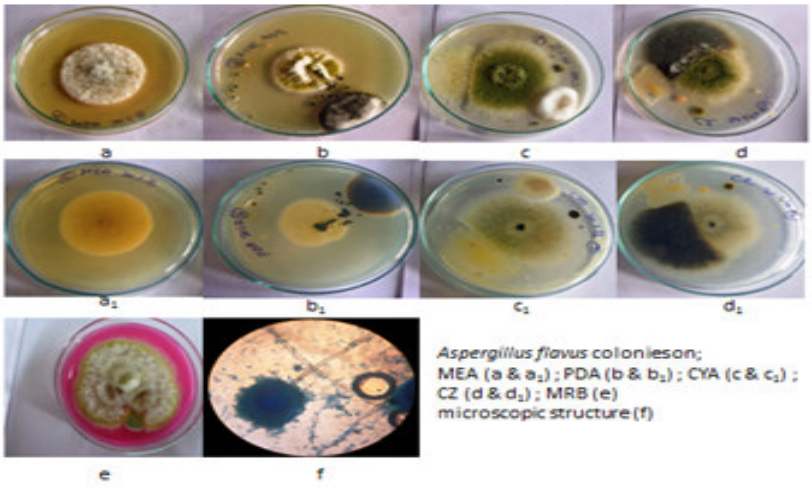

Figure 5
Aspergillus flavus colonies and microscopic structure

DISCUSSION

All the results were recorded within a week period. The *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus niger, Aspergillus violaceofuscus, Aspergillus neoniger, Rhizopus*[13] *and Penicillium* isolated in the present study are reported to be the most common fungal contaminant of grains. The presence of fungal hyphae and spores can be found in infected grains which may change the colour and form of grains. But the presence of *Aspergillus* growth in food crop need not necessarily indicate mycotoxin production.[14] Among the different *Aspergillus species* obtained, the flavus species was found to be predominating in this study. It was seen that all infection in peanut and corn samples in the present study were due to *Aspergillus* species only and other fungal species were not identified when tested in the basal medium for fungi. Though the soil is a habitat for many types of fungal strains, only selected fungi have the ability to establish infection in the food crop as such. The food crop contamination occurs not only from the field soil contamination but also by many other sources like field instruments, insects, pests, rodents or birds and also during the food storage process. The presence and growth of *Aspergillus* on pre-harvested crops is dependent on the environmental factors. The wide spread occurrence of *Aspergillus flavus* show the extent of pre-harvest and post- harvest contamination occurring naturally.[15] Among the *Aspergillus species* obtained in the present study, the predominating fungal species was *A. flavus* (63%) followed by, *Aspergillus niger* (16%), *Aspergillus neoniger* (13%), *Aspergillus*

violacefuscus (6%) and *Aspergillus parasiticus* (2 %). This indicates that the main aflatoxin contamination in food grains could be due to *Aspergillus flavus*. All the isolated *Aspergillus flavus* and *Aspergillus parasiticus* were found to be aflatoxin producers. Among the*Aspergillus* isolates found in peanut and corn samples 65% of isolates belonging to either *Aspergillus flavus* or *Aspergillus parasiticus sp.* producing aflatoxin. The 65% aflatoxin producing *Aspergillus* species in corn comprised mostly the *Aspergillus flavus* (60%) with *Aspergillus parasiticus* (5%). In the present study the percentage of aflatoxin producing species was equally distributed between the corn and peanut samples eventhough the *A. parasiticus* was isolated from corn alone. The percentage of aflatoxigenic strains of *Aspergillus flavus* has been shown to vary with the nature of substrate and environmental factors. [16, 17] For example, the incidence of aflatoxigenic *Aspergillus flavus* strains was higher in peanuts (69%) than in wheat (13%) in the study made byVaamonde*et al.*[18] Mohammed *et al.,*[19] had found *Aspergillus flavus* contamination in peanut kernels ranging from 20% to 48% varying with the region and place of sample collection. They also reported higher incidence of *Aspergillus flavus* species in peanut samples from storage godowns when compared to *Aspergillus parasiticus* species among all the study samples made. The study results confirm that the growth of fungal species depends on both intrinsic and extrinsic factors.In the present study the aflatoxin producing *Aspergillus flavus* were isolated from the affected corn and peanut samples. Also *Aspergillus parasiticus* were isolated from corn samples indicating that there is a danger of mycotoxins contamination in the food and feed products prepared out of contaminated nuts and kernels. The food grains with comparatively little water activity were found to have much *Aspergillus* aflatoxin contamination when compared to the fleshy plant crops contaminated or inoculated with the same organisms.[18] Complexes of pathogenic and opportunistic species of *Aspergillus* can colonise and induce disease symptoms in various plants and plant products, and produce toxic secondary metabolites (mycotoxins) in the infected tissue. *Aspergillus flavus, Aspergillus parasiticus* remain the most important and representative aflatoxin producers occurring naturally in food commodities like peanut oil mills and corn storage godowns. Though aflatoxin quantification is not a part of this study, from literatures it is evidential that among *Aspergillus* isolates, there is great variation in aflatoxin production especially within the most common aflatoxin-producing species, *Aspergillus flavus*.[20] Detection of mycotoxin producing fungi no matter what the level or intensification of production may be, is a discouraging sign and a warning for food and feed storage godowns. Agricultural interventions are methods or technologies that can be applied either in the field (pre harvest) or in drying, storage and transportation (post-harvest) to reduce aflatoxin levels in food.[21] Agricultural practices including proper irrigation and pest management can reduce aflatoxin contamination. Pre-harvest interventions include choosing crops with resistance to drought, disease, and pests and choosing strains of that crop which are genetically more resistant to the growth of the fungus and the production of aflatoxin.During storage process the moisture, insect and rodent control can prevent damage to the stored food grains and reduce aflatoxin development.As a prevention strategy, the godown can be completely subjected to thorough cleaning process using aseptic and sterilization procedures. Also during sunny days fresh food or feed grains should be dried to optimum level before their next storage process. During winter seasons some biocontrol[22] measures applying antifungal phytoproducts in congruence to safety standards can be adopted.

CONCLUSION

The study used simple cost effective fungal and mycotoxin identification techniques quick and easy to perform. The present study showed the occurrence of aflatoxin producing strains in food crops namely corn and peanuts stored in godowns intended to be used for food and fodder preparation. The study showed that earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques. Their earlier detection may help to adopt physical management practicesand to initiate some biocontrol methods to avoid mycotoxic contamination.

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