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Screening of *Penicillium* from *Arachis hypogea* and its Mycotoxin Production

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Abstract

Seed infections by *Penicillium* not only reduce seed quality but also make nuts unsafe for human consumption. Direct plate method using CDA (Czapek Dox Agar) medium was employed for isolation of *Penicillium*, the total no. of colony forming (CFU) units observed in plate were 13. *Penicillium* strain was identified by Lactophenol cotton blue staining. The *Penicillium* was inoculated into CD broth to measure its growth as well as mycotoxin production at different duration as 5, 10, 15, 20 days. Paper chromatography was used to identify amino acid in metabolite as Histidine. Mycotoxin production was confirmed by Thin Layer Chromatography (TLC) and a spot was identified, which was blue in visible light and faint blue under UV light. This indicated the presence of Aflatoxin B2 in culture metabolite when compared with standard. ELISA was also performed to assess the presence of antibodies against fungus and also used for the detection of mycotoxin level in culture metabolite. From the standard curve it was observed that serum sample had no antibodies for *Penicillium* and the patient was free from the infection of *Penicillium* as the level of mycotoxin in culture metabolite was low.

Keywords: *Penicillium*; *Arachis hypogea*; Seed quality; Thin Layer Chromatography (TLC); Aflatoxins; Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Ground nut (*Arachis hypogea*) is the sixth most important oilseed crop in the world. It is grown on 23.52 million hectares worldwide with a total production of 38.38 million metric tonnes and an average productivity of 1634 kg/ha [1]. Over 100 countries worldwide grown groundnut and due to its economic and nutritive value, this crop is a significant enterprise. Seed infections by mycotoxin not only reduce seed quality but also make nuts unsafe for human consumption. Molds such as (*Mucor, Rhizopus*, *Aspergillus*, *Penicillium* and *Botrytis* etc.) lead to deterioration of food. Some molds are harmful and produce toxic metabolites Mycotoxins, others are mutagenic and carcinogenic. Species of *Penicillium* are ubiquitous soil fungi preferring cool and moderate climates, commonly present wherever organic material is available. The ability of these *Penicillium* species to grow on seeds and other stored foods depends on their propensity to thrive in low humidity and to colonize rapidly by aerial dispersion while the seeds are sufficiently moist. *Penicillium* spp. occasionally caused infection in humans being known as Penicilliosis. Most *Penicillium* infections are encountered in immunosuppressed hosts. The following toxins: aflatoxins B1, B2, G1, and G2, Ochratoxin A, aspertoxin, luteoskyrin, zearalenone, 4-acetamido-4- hydroxy-2-butenoic acid y-lactone, diacetoxyscirpenol and its 8-(3-methylbutyryloxy) derivative and nivalenol and its 4-0-acetate, gliotoxin, citrinin, patulin, penicillic acid, and sterigmatocystin were detected. These mycotoxins are produced mainly by species of *Aspergillus*, *Penicillium* or *Fusarium* but are not necessarily restricted to any one species or genus [2]. Mycotoxins as secondary metabolites produced by fungi may have developed to serve as a chemical defense system against insects, microorganisms, nematodes, grazing animals and humans. Approximately 400 known mycotoxin exist. Mycotoxins can benefit humans by their use as antibiotics (Penicillin), immunosuppressant (cyclosporine), and in control of postpartum hemorrhage and migraine headaches (ergot alkaloids). But the problem arises in attempting to assess the effect of extremely small amount of very toxic chemicals in the diet. Reasons why mycotoxins identification is important are suggested as their risk to human health and effect on human health, impact on livestock production and productivity, legislation (when it exists), demands of the food industry for high quality raw materials and concern expressed by the public or media. Keeping these points in view, the present study was designed to detect the nature of mycotoxin produced by *Penicillium* in *Arachis hypogea* (Groundnut).

Materials and Methods

Infected groundnut seeds were obtained from local market and isolation, identification and screening of mycotoxins were carried out.

Isolation of fungi

For isolation of fungi, CDA (Czapek Dox Agar) medium were prepared. Each CDA plates were inoculated with four surface sterilized seeds. Then plates were incubated at 28°C for 6-8 days.

Identification of fungi

Lactophenol cotton blue staining was used for identification. Put a drop of lactophenol cotton blue stain in the centre if glass slide. A portion of mycelia mat from fungal colony was transferred into the drop of stain with the help of needle. Gently spread the fungal propagates so that mycelia get mixed with the stain [3]. Carefully put the cover slip on the slide carefully so that no air bubble appeared. Finally observed the slide under microscope.

Measurement of fungal growth

Fungal growth was measured by biomass method. Prepared Czapek Dox Broth medium and sterilized at 15 psi/inch. Cut agar block from actively growing margin of *Penicillium* by using a sterile cork borer. Transferred the agar block into flask. Incubated the flask at 28°C.

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Filtered the mycelia mat through pre weight whatman No.1 filtered paper and dried at 80°C for 24 hours. Weighed dried mycelia and recorded the dry weight [4].

Detection of amino acid by paper chromatography

Took the sample extract and loaded it on a line 2.5cm away from one end of the paper. Kept the paper strip in solvent system containing Butanol: Acetic acid: H_2O (4:1:5). After sometime when solvent reached to maximum level on strip, took out from solvent system. Air dried the paper and then dips the paper strip in ninhydrin solution. Kept it in oven for minute and observed the spots on paper strip.

Extraction and identification of mycotoxin by thin-layer chromatography (TLC)

Preparation of mycotoxin extract-Filtered the medium having growth of *Penicillium* through Buchner funnel using whatman filter paper. Extract 100.0 ml of the culture filtrate thrice with equal volume of chloroform. Pool the chloroform extract and concentrated it to dryness in a rotator. Dissolved the residue in minimum quality of distilled water.

Preparation of plate

Weighed 30.0g of silica gel G, added 60-65ml distilled water to form slurry and transferred to the applicator for spreading on clean glass slide. Dried the plate and kept it at 100°C for 30 minutes before use. Took sample extract and loaded it on line 2-5cm away from one. Kept the plate in solvent system containing Acetone: Chloroform (12:88) (v/v) for 10 minute. Took out the plate and kept it at room temperature for overnight. Examine the plate under long wave UV light [2].

Enzyme linked immune sorbent assay (ELISA)

Add 10 µl sample into 1000 µl dilution RF dilution buffer. Add 50 µl of above dilution into 200 µl dilution buffer (200 µl RF absorbent+80

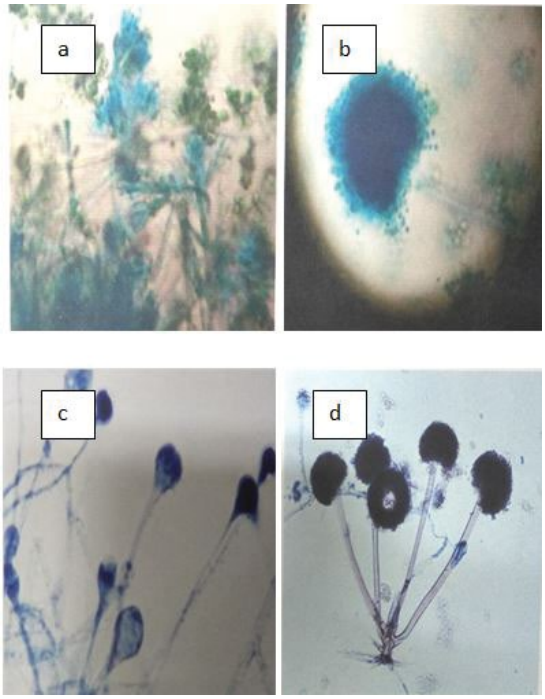

Figure 1: Microscopic structure of fungi isolated from *Arachis hypogea* (a) *Penicillium* (b) *Aspergillus* (c) *Mucor* (d) *Rhizopus*

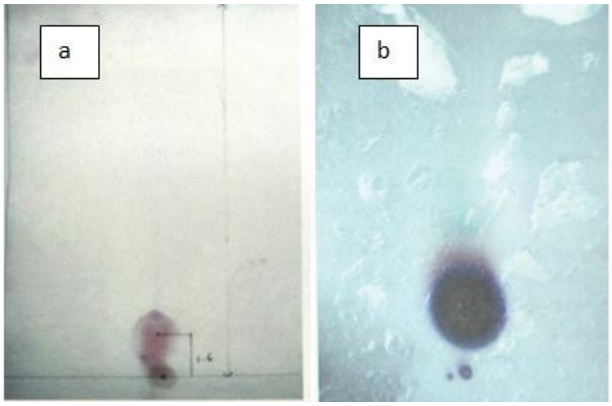

Figure 2: (a) Paper chromatography of cultural metabolite produced by *Penicillium* (b) Thin layer chromatography of cultural metabolite produced by *Penicillium*.

Fungi	Cfu ± SEM
Penicillium	4 ± 0.38
Aspergillus	6 ± 1.154
Mucor	1 ± 0.38
Rhizopus	2 ± 1.535
Cfu: Colony Forming Unit, SEM: Standard Error Mean	

Table 1: Isolation of fungi from *Arachis hypogea* by direct plate method (Average of triplicates).

Fungi	Colony colour	Hyphae	Spores
Penicillium	Blue	Septate	Conidium long chains on repeatedly branched conidiophores.
Aspergillus	Lime-green	Septate	Conidia on phialides on vesicle formed by condiophores.
Mucor	White to dark grey	Aseptate mycelium	Spores present in black columellate singly
Rhizopus	White to dark grey	Aseptate mycelium with rhizoids	Spores present in black columellate in cluster.

Table 2: Identification of fungi by fungal staining method using lactophenol cotton blue stain.

Incubation time (days)	Dry weight (gm) ± SEM	O.D at 420nm
5	0.836 ± 0.989	0.015
10	1.089 ± 1.002	0.096
15	0.947 ± 0.994	0.267
20	0.126 ± 0.230	0.199

Table 3: Showing growth of *Penicillium* after different interval of days (Average of triplicates).

Sample	Rf value(experimental)	Rf value (standard)	Amino acid
Culture metabolite	0.11	0.11	Histidine
Culture metabolite-After 10 days of incubation Rf- Distance travelled by solute/ distance travelled by solvent			

Table 4: Detection of amino acid in culture metabolite of *Penicillium* by paper chromatography method.

Sample	Rf value	Fluorescence		Mycotoxin
		Visible light	Visible light	
Culture metabolite	0.11	Blue	Faint blue	Aflatoxin B2
Rf-Distance travelled by solute/distance travelled by solvent Culture metabolite-After 10 days of incubation				

Table 5: Extraction and Identification of mycotoxin by thin layer chromatography.

Negative	000.42
Test serum	000.19
Culture metabolite	000.25
Culture metabolite-After 10 days of incubation	

Table 6: ELISA for serum/metabolite sample.

µl dilution buffer). Pipette diluted sample and ready to use control sera/ standard sera into the micro testwell (100 µl). Incubated it at 37°C for 60 minutes. Wash it with washing solution. Pipette conjugate solution (100 µl). Incubated it at 37°C for 30 minutes. Wash it with washing solution. Pipette standard solution (100 µl). Incubated it at 37°C for 30 minutes. Pipette stopping solution (100 µl). Took absorbance at 405 nm [5].

Results and Discussion

In the present study the peanut was found contaminated with different type of fungi include *Penicillium*, *Aspergillus*, *Mucor*, and *Rhizopus*. *Penicillium* strain identified by lactophenol cotton blue staining method (Tables 1 and 2) and (Figure 1) similar finding were observed [6]. The fungal (*Penicillium*) growth as well as mycotoxin production was measured at different duration as 5, 10, 15, and 20 days (Table 3). The amino acid detected in metabolite produced by *Penicillium* by paper chromatography method was Histidine (Table 4) and (Figure 2a). *Penicillium* produces secondary metabolite (Mycotoxin) which was detected by TLC method. One spot observed on TLC plate which was blue in visible light and faint blue in UV light (Table 5 and Figure 2b) which concurs with the findings reported [2]. The colours detected in this are same as that observed in present study therefore on the basis of this we can say that the isolated mycotoxin was Aflatoxin B2. Corroborated finding was also reported [7]. Rf values detected by them are almost same as that observed in this study therefore on the basis of this we can say that the isolated mycotoxin was Aflatoxin B2. Thin layer chromatography for mycotoxin detection also carried out by various workers [8] where they concluded that as the worldwide requirement for the control analysis of mycotoxins in various commodities is increasing. Previous study [9] have shown that many chromatographic methods used for the detection of mycotoxins produced by species of *Penicillium*, *Aspergillus*, and *Alternaria* and concluded that the TLC methods that predominated in early 1970s have given way to methods based on Liquid chromatography (LC). Chromatographic methods (TLC, HPLC) for routine analysis of mycotoxins were used [10]. The detection of mycotoxins fungi and their toxin production ability to stored grains deteriorate the quality of stored produce. Hence the present study carried out to assess major fungal types and their specificity of mycotoxin production [11]. Frequently isolated mycotoxin fungi lowering seed quality and this could be due to its evolutionary advantage of massive gene swapping [12]. ELISA also used for the confirmation of presence of antibodies against fungus. This is also used for the detection of mycotoxin level in culture metabolite (Table 6) similar test was done [5] where method for mycotoxin detection was also performed.

Conclusion

Finally we concluded that *Penicillium* mainly contaminate the food (maize, peanut, rice) during their storage and produces highly toxic mycotoxins in it. Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. These toxins can also be transferred to the food products from its raw food. So there are centers for food safety, which work to protect human health and the environment by curbing the proliferation of harmful food production technologies and by promoting organic and other forms of sustainable agriculture. For the future course of action to reduce contamination, it is necessary to introduce a system to check this problem. Efforts have been made to develop mycotoxin resistant transgenic peanut plant. This can be an effective long term approach to the problem. Mould inhibitor can be used for effective conservation and control of mycotoxin production. This can be done along with the education to the farmers, grain handlers and marketing people for exchanging safety and minimizing loss. To conclude contamination commonly used food and feed is an important unrecognized risk to public health and can have long term health implications. Thus to ensure grains and legume remain free of fungal infections, certain conditions must be incorporated before, during and after harvest. Moisture level should be kept as low as 11.5% temperature should also be kept low. The most common management practice for grain and legumes is through the use aeration system. Several antifungal drugs like Amphoterecin B, itraconazole, fluconazole etc are used for the control of fungal contamination wide-spreadly.

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