International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 6(6): November-December 2016

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Life Sciences

Research Article……!!!

Received: 17-10-2016; Revised: 31-10-2016; Accepted: 01-11-2016

SCREENING OF *PENICILLIUM* FROM *ARACHIS HYPOGEA* & ITS MYCOTOXIN PRODUCTION

Singh Padma* & Prasad Richa

Department of Microbiology, Kanya Gurukul Campus, Gurukul Kangri University, Haridwar-249407, Uttrakhand, INDIA

Keywords:

Penicillium, Arachis hypogea, Seed quality, thin layer chromatography (TLC), Aflatoxins, enzyme-linked immunosorbent assay (ELISA)

For Correspondence:

Dr. Singh Padma

Department of Microbiology, Kanya Gurukul Campus, Gurukul Kangri University, Haridwar-249407, Uttrakhand, INDIA

E-mail:

dr.padmasingh06@gmail.com

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) & a spot was identified, which was blue in visible light & faint blue under UV light. This indicated the presence of Aflatoxin B_2 in culture metabolite when compared with standard. ELISA was also performed to assess the presence of antibodies against fungus & 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* & the patient was free from the infection of *Penicillium* as the level of mycotoxin in culture metabolite was low.

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 & an average productivity of 1634 kg/ha [1]. Over 100 countries worldwide grown groundnut & due to its economic & 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* & *Botrytis* etc.) lead to deterioration of food. Some molds are harmful & produce toxic metabolites Mycotoxins, others are mutagenic & carcinogenic. Species of *Penicillium* are ubiquitous soil fungi preferring cool & moderate climates, commonly present wherever organic material is available. The ability of these *Penicillium* species to grow on seeds & other stored foods depends on their propensity to thrive in low humidity & 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 B_1, B_2, G_1, & G_2, Ochratoxin A, aspertoxin, luteoskyrin, zearalenone, 4-acetamido-4-hydroxy-2-butenoic acid y-lactone, diacetoxyscirpenol & its 8-(3-methylbutyryloxy) derivative & nivalenol & its 4-0-acetate, gliotoxin, citrinin, patulin, penicillic acid, & 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 & humans. Approximately 400 known mycotoxin exist. Mycotoxins can benefit humans by their use as antibiotics (Penicillin), immunosuppressant (cyclosporine), & in control of postpartum hemorrhage & 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 & effect on human health, impact on livestock production & productivity, legislation (when it exists), demands of the food industry for high quality raw materials & concern expressed by the public or media. Keeping this in mind, the present study carried out to detect the nature of mycotoxin produced by *penicillium* in *arachis hypogea* (Groundnut).

MATERIALS & METHODS

Infected groundnut seeds were obtained from local market & isolation, identification & 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 propagules 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 & 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. Filtered the mycelia mat through pre weight whatman No.1 filtered paper & dried at 80°C for 24 hours. Weighed dried mycelia & recorded the dry weight.

Detection of amino acid by paper chromatography- Took the sample extract & 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 & then dips the paper strip in ninhydrin solution. Kept it in oven for minute & observed the spots on paper strip.

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

Preperation 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 & concentrated it to dryness in a rotator. Dissolved the residue in minimum quality of distilled water.

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

minute. Took out the plate & kept it at room temperature for overnight. Examine the plate under long wave UV light [2].

Enzyme linked immune sorbent assay (ELISA)-Add 10µl sample into 1000µl dilution RF dilution buffer. Add 50µl of above dilution into 200µl dilution buffer (200µl RF absorbent+80µl dilution buffer). Pipette diluted sample & ready to use control sera/standard sera into the micro testwell (100µl). Incubated it at 37°C for 60 minutes. Wash it with washing solution. Pipette conjugate solution (100µl). Incubated it at 37°C for 30 minutes. Wash it with washing solution. Pipette standard solution (100µl). Incubated it at 37°C for 30 minutes. Pipette stopping solution (100µl). Took absorbance at 405 nm [4].

RESULTS & DISCUSSION

In the present study the peanut was found contaminated with different type of fungi include *Penicillium, Aspergillus, Mucor,* & *Rhizopus. Penicillium* strain identified by lactophenol cotton blue staining method (Table 1, 2) & (Fig. 1) similar finding were observed [5]. The fungal (*Penicillium*) growth as well as mycotoxin production was measured at different duration as 5, 10, 15, & 20 days (Table 3).The amino acid detected in metabolite produced by *Penicillium* by paper chromatography method was Histidine (Table 4) & (Fig. 2A). *Penicillium* produces secondary metabolite (Mycotoxin) which was detected by TLC method. One spot observed on TLC plate which was blue in visible light & faint blue in UV light (Table 5) & (Fig. 2 B) 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 B_2. Corroborated finding was also reported [6]. 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 B_2. Thin layer chromatography for mycotoxin detection also carried out by various workers [7] where they concluded that as the worldwide requirement for the control analysis of mycotoxins in various commodities is increasing. Previous study[8] have shown that many chromatographic methods used for the detection of mycotoxins produced by species of *Penicillium, Aspergillus*, & *Alternaria* & 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 [9]. The detection of mycotoxins fungi & their toxin production ability to stored grains deteriorate the quality of stored produce. Hence the present study carried out to assess major fungal types &

their specificity of mycotoxin production [10]. Frequently isolated mycotoxin fungi lowering seed quality & this could be due to its evolutionary advantage of massive gene swapping [11]. 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 [4] where method for mycotoxin detection was also performed.

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

S.No.	Fungi	Cfu±SEM
1.	*Penicillium*	4±0.38
2.	*Aspergillus*	6±1.154
3.	*Mucor*	1±0.38
4.	*Rhizopus*	2±1.535

Cfu= colony forming unit, SEM=standard error mean

Table2. Identification of fungi by fungal staining method using lactophenol cotton blue stain.

S.No.	Fungi	Colony colour	Hyphae	Spores
1.	*Penicillium*	Blue	Septate	Conidium long chains on repeatedly branched conidiophores.
2.	*Aspergillus*	Lime-green	Septate	Conidia on phialides on vesicle formed by condiophores.
3.	*Mucor*	White to dark grey	Aseptate mycelium	Spores present in black columellate singly.
4.	*Rhizopus*	White to dark grey	Aseptate mycelium with rhizoids	Spores present in black columellate in cluster.

Table3. Showing growth of *Penicillium* after different interval of days (Average of triplicates)

S.No.	Incubation time (days)	Dry weight (gm)±SEM	O.D at 420nm
1.	5	0.836±0.989	0.015
2.	10	1.089±1.002	0.096
3.	15	0.947±0.994	0.267
4.	20	0.126±0.230	0.199

Table4. Detection of amino acid in culture metabolite of *Penicillium* by paper chromatography method

S.No.	Sample	Rf value(experimental)	Rf value (standard)	Amino acid
1.	Culture metabolite	0.11	0.11	Histidine

Culture metabolite- After 10 days of incubation

Rf- Distance travelled by solute/distance travelled by solvent

TABLE 5: Extraction & Identification of mycotoxin by thin layer chromatography

S.No.	Sample	Rf value	Fluorescence		Mycotoxin
			Visible light	UV light	
1.	Culture metabolite	0.11	Blue	Faint blue	Aflatoxin B_2

Rf-Distance travelled by solute/distance travelled by solvent

Culture metabolite-After 10 days of incubation

TABLE 6: ELISA for serum/metabolite sample

S.No.	Sample	Absorbance at 405nm
1.	Black	000.00
2.	Standard	000.34
3.	Negative	000.42
4.	Test serum	000.19
5.	Culture metabolite	000.25

Culture metabolite-After 10 days of incubation

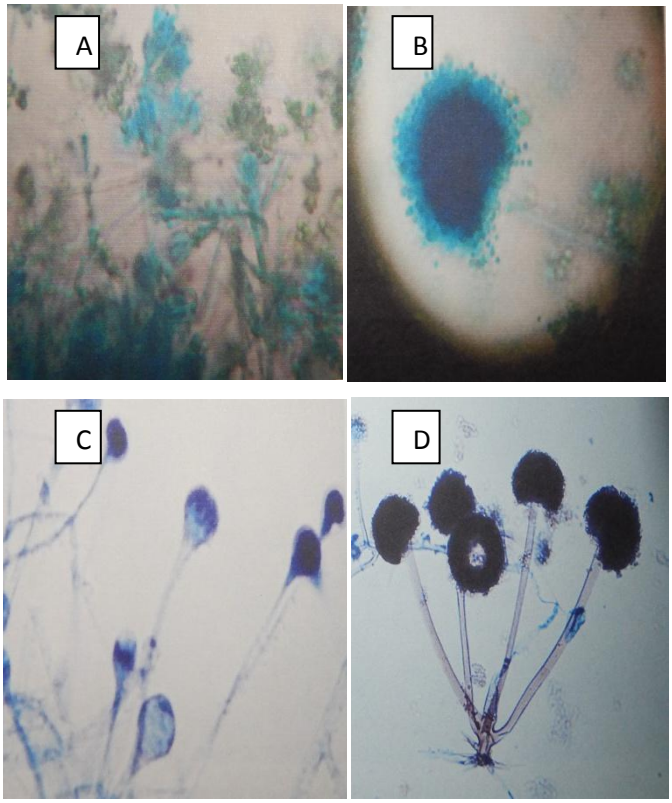

Fig: 1 Microscopic structure of fungi isolated from *Arachis hypogea* (A) *Penicillium* (B) *Aspergillus* (C) *Mucor* (D) *Rhizopus*

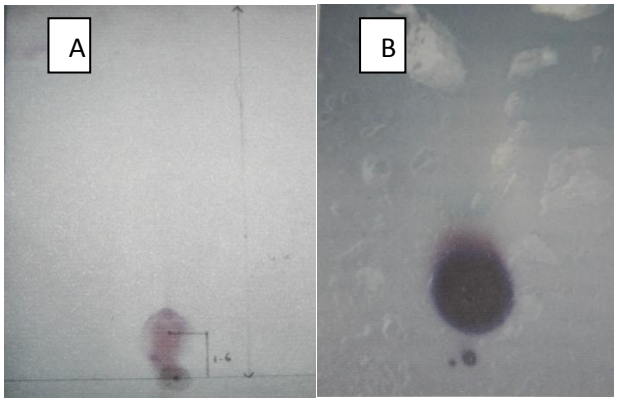

Fig: 2 (A) Paper chromatography of cultural metabolite produced by *Penicillium*. (B) Thin layer chromatography of cultural metabolite produced by *Penicillium*.

CONCLUSION

Finally we concluded that *penicillium* mainly contaminate the food (maize, peanut, rice) during their storage & produces highly toxic mycotoxins in it. Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling & in storage. These toxins can

also be transferred to the food products from its raw food. So there are centres for food safety, which work to protect human health & the environment by curbing the proliferation of harmful food production technologies & by promoting organic & 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 & control of mycotoxin production. This can be done along with the education to the farmers, grain handlers & marketing people for exchanging safety & minimizing loss. To conclude contamination commonly used food & feed is an important unrecognized risk to public health & can have long term health implications.

Thus to ensure grains & legume remain free of fungal infections, certain conditions must be incorporated before, during & 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 & legumes is through the use aeration system. Several antifungal drugs like Amphoterecin B, itraconazole, fluconazole etc are used for the control of fungal contamination widespreadly.

REFERENCES

1. FAO Stat. Statistical database. Food and Agriculture Organization (FAO). Available online: http://www.faostat. fao.org, 2011.
2. Scott PM., Lawrence JW., Walbeek WV., Detection of mycotoxins by thin-layer chromatography: Application to screening fungal extracts, Applied microbiology 1970, 20(5): 839-842
3. Barnett HL., Manual for Hypomycetes fungi, The APS, St. Paul, Minnesota 55121, USA, 2003.
4. Zheng MZ., Richard JL., Binder Johann, A review of rapid methods for the analysis of mycotoxins, Mycopathologia, 2006, 161(5): 261-273.
5. Khosravi AR., Shokri H., Ziglasi T., Evaluation of fungal flora in some important nut products in Tehran, Iran, Pakistan (J) of nutrition, 2007, 6(5): 460-462.
6. Robb J., Norval M., Comparison of cytotoxicity & Thin-layer chromatography methods for detection of mycotoxins, App. & environmental microbiology, 1983, 46(4): 948-950.
7. Lin L., Zhang J., Wang P., Wang Y., Chen J., Thin layer chromatography of mycotoxins & comparison with other chromatographic methods, Journal of chromatography A, 1998, 815(1): 3-20.
8. Barkai-Golan R., Paster N., Mouldy fruits & vegetables as a source of mycotoxins, World mycotoxin journal, Wageningen Academic publishers, 2008, 1(2): 147-159.
9. Vosough M., Bayat M., Salemi A., Matrix free Analysis of Aflatoxin in pistachio nuts using parallel factor modelling of liquid chromatography, Analytica chimica acta, 2010, 663(1): 11-18.
10. Rao VK., Girisham S., Reddy SM., Incidence of mycotoxigenic Penicillia in feeds of Andhra Pradesh, India, International Journal for Biotechnoloy & Molecular Biology Research, 2011, 2(2): 46-50.
11. Ropars J., Aguileta G., deVienne DM., Giraud T., Massive gene swamping among cheese-making Penicillium fungi, Microbial cell, 2014, 1(3): 107-109.