February 2013- April 2013, Vol. 3, No. 2, 1327-1337. E- ISSN: 2249 –1929

Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section B: Biological science

CODEN (USA): JCBPAT

Research Article

Comparison of microscopic, macromorphological and aflatoxin producing capabilities of *Aspergillus* species associated with rhizosphere of groundnut (*A. hypogaea* L.)

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Received: 7 March 2013; **Revised:** 22 April 2013; **Accepted:** 31 April 2013

Abstract: The role of laboratory in the identification has gained greater attention as the incidence of fungal infection is increased. The objective of the study was to isolate the aflatoxin producing *Aspergillus* species from the rhizosphere of groundnut. Identification of the *Aspergillus* species was done on the basis of morphological methods including; microscopic and macroscopic characteristics. Ten standard species of *Aspergillus* namely *A. flavus, A. parasiticus, A. niger, A. repens, A. fumigatus, A. kanagawaensis, A. ochraceus, A. sydowi, A. terreus and A. nidulans* and were isolated and characterized. Rapid detection of aflatoxin production was carried out by using coconut agar medium. The colonies were detected under long-wave UV light by the blue-green fluorescence on the reverse side after 2 to 5 days of growth. Further the aflatoxin production capacity was screened using thin layer chromatographic technique. Depending upon the intensity of bands on TLC plates *A. flavus* and *A. parasiticus* were found to be highly aflatoxigenic and produce aflatoxin B_1 on Richard liquid medium in the range of 966.66-266.66 µg/kg respectively. Quantitation of aflatoxin was done by using quantitative TLC, which was based on the comparison of the fluorescent intensity of the sample spot with standard spot. While all the other species are non-aflatoxigenic strains.

Keywords: Aflatoxin, TLC, groundnut rhizosphere fungi, microscopic and macroscopic characterization

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important and unique legume crop in many tropical and subtropical areas of the world[14]. It produces seed-bearing pods below the soil surface. Pods are in direct and seeds in indirect contact with soil fungal populations before harvesting. Infection of peanut by *Aspergillus* species, especially *A. flavus* and *A. parasiticus* group occurs under both pre-harvest and post-harvest conditions[9].

Aflatoxin is one of the mycotoxins produced by the genus *Aspergillus*[24]. The aflatoxin producing fungi infest a number of food crops, most notably corn and groundnut. The genus *Aspergillus* is one of the most widely studied genera of spoilage fungi associated with nuts, grain and seed crop products[24]. This genus composed of more than 180 accepted anamorphic species, with telemorphs described in nine different genera. It is economically as well as ecologically important fungi in industry and many fields of applied research.

These mycotoxins have been reported to be accurately toxic, teratogenic and potent carcinogenic and mutagenic agent[25]. Incidence of aflatoxin contamination in groundnut grown in tropical areas of the world is generally much higher.

In identification of *Aspergillus* species, molecular and biomolecular methods are developed and rapidly available while microscopy and cultural characteristics remains commonly used and essential tools for identification of *Aspergillus* species[5]. The species level identification is very complex. Macromorphological identification is done based on conidial and mycelial color, colony diameter, colony reverse color, production of exudates and soluble pigments. Microscopic identification is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology[15]. The spores come in several colors, depending upon the species and are produced in long chain from the ends the phialides[2].

In this study we emphasize on macroscopic characteristics of colonies and microscopic features for identification of *Aspergillus* species isolated from the rhizosphere of groundnut. To identify the aflatoxin producing *Aspergillus* species microscopic identification alone may be misleading. Thus for accurate determination of aflatoxin producing ability of a particular *Aspergillus* species, coconut agar screening medium was used, which is based on the detection of blue fluorescence under UV light and orange-yellow pigmentation of particular colony[16, 4]. The capacity to secrete aflatoxin *in vitro* and its analysis were carried out by using thin layer chromatographic technique. The work was also sought to determine the concentration of aflatoxinB_1 by using quantitative TLC in which aflatoxins are extracted from the substrates with organic solvent, concentrated and assayed.

A comparative study on the influence of various culture media along with modification in Czapeks – Dox media on the production of aflatoxins by two selected species of *A. flavus* and *A. parasiticus* was carried out under surface fermentation.

EXPERIMRNTAL

Our major subject was to isolate aflatoxigenic *Aspergillus* species from rhizosphere of groundnut. The soil samples were isolated from the different sites of Akola and Amravati district (MS) India.

Isolation of Soil Fungi (soil dilution Method): For collection of rhizosphere soil sample, plants were carefully dugout with roots; excess soil was gently shaken off and discarded, leaving only the soil closely adhered to the root system.

Roots were further placed in screw cap bottle containing 100mL sterile distilled water and shaken well in a rotating motion for 10 min for soil dilution or soil washing. Serial dilutions method was

used for isolation. One milliliter of an appropriate dilution was transferred into sterile five Petri dishes and Rose Bengal Agar medium (12-15 ml) just above the solidifying was poured. The dishes were rotated by hands to complete homogeneity, incubated at 28 ± 2° C for 7-15 days and the developing fungi were identified and counted per gram fresh weight of roots of each tested sample.

Stock culture preparation of fungal isolates: *Aspergillus* isolates were identified in the level of genus on Rose Bengal Agar. To improve the sensitivity and specificity of culture approach for identification of Aspergilli in the level of species, four differential media were used. These including, Potato Dextrose Agar (PDA){potato infusion- 200g, dextrose-20g, agar-17g, water distilled 1000mL}, Czapek's Yeast Agar (CzYA) {$NaNo_3$-30g, $MgSO_4.7H_2O$-5g, $FeSO_4.7H_2O$-100mg, KCL-5g, Czapek's concentrate keeps indefinitely without sterilization. K_2HPO_4–1g, Czapek's concentrate-10ml, Yeast extract–5g, Sucrose–30g, Agar–15g, Water(distilled)-1000ml}, Czapek's solution agar (CzSA) {$NaNO_3$–2g, K_2HPO_4–1g, $MgSO_4.7H_2O$-0.5g, KCL–0.5g, $FeSO_4.7H_2O$–100mg, Sucrose–30g, Agar-15g, water(distilled)- 1000ml}, Rose Bengal agar (RBA) {dextrose– 10.33g, Yeast extract–0.50g, K_2HPO_4–0.60g, $MgSO_4.7H_2O$–0.50g, Peptone-0.50g, Rose Bengal–50mg, Agar- 17g, Streptomycin- 30mg, water (distilled) – 1000ml}.

Characterization and Identification of fungal Isolates: Taxonomic identification of fungi (based on purely morphologically micro- and macroscopic characteristics) was carried out according to International Scientific Mycological References. The major and remarkable macroscopic features in species identification were the colony features including diameter after 7 days, color of conidia, mycelial color, colony reverse, colony texture and shape.

For microscopic study, Lacto-phenol cotton blue staining procedure was used. For the proper visualization of characteristic features, slides were prepared from older colonies, because they were covered with too many spores. However, for examination of characters of spores the slides were prepared from two weeks old culture. Microscopic characteristics for the identification were conidial head, conidia shape, roughness and vesicle serration were taken into consideration.

Cultivation and observation of fluorescence: Readily available local brand of coconut were purchased locally, 100g of shredded coconut was homogenized for 5 min in 100 ml of distilled water. The homogenate was filtered through muslin cloth and the pH of the filtrate was adjusted to pH 7 with 2 N NaOH, 20g of agar was added and the mixture was heated upto boiling. The pH was again checked and adjusted to 7 and the mixture was then autoclaved[4].

Each mycelial plug of *Aspergillus* isolates was placed on the centre of plate containing coconut agar medium and incubated for 8 days. Plates were then examined under UV light for fluorescence.

Sample Preparation for *in vitro* Aflatoxins production: For evaluation of aflatoxin production, the six different liquid medium viz. Richard broth, SMKY liquid medium, Czapek-Dox liquid medium along with the modification (8.6% glucose + 0.4% citric acid), yeast extract (2%+ sucrose 10%) and yeast extract (2%+ sucrose 20%) was used. The 50 ml of medium with pH 5.5 was prepared routinely in 250 ml Erlenmeyer flask and culture plug of the isolates was inoculated aseptically. Triplicates were kept at 26 ± 2°C for 9 days.

Aflatoxin extraction (cleanup of crude extract): Cell-free culture filtrate was obtained by separating mycelial mat through Whatman filter paper. Aflatoxin was extracted in chloroform by repeated fractionation. Chloroform extract was then passed through a bed of anhydrous sodium sulphate. Further, the extract was concentrated by evaporating on water bath at 40°C. Final residue was redissolved in 100µl chloroform and used for TLC.

Thin layer chromatography: For qualitative analysis of aflatoxin, thin layer chromatography technique was employed. 5µl of extracted sample were spotted on glass plates coated with silica gel G. The spots were resolved in a solvent system comprising Toulene : Acetonitrile (9:1, v/v) (AOAC 2000), dried and the blue-green fluorescence of aflatoxin was observed under UV light at 365nm for fluorescence and compared with the standard.

Quantitative TLC: Aflatoxin quantitation was done by visual comparison of fluorescence intensity of standard spot with that of sample spot on TLC plate. Final residue was redissolved in Toulene : Acetonitrile (9:1) in known volume. Test solutions were successively spotted on TLC plate in the range of 3, 4 and 5ul in equal diameter. Along with test solution 5ul standard (10ug/ml) was loaded on the same plate. The plate was then allowed to run in chamber having saturated solvent system (Chloroform : Acetone, 9:1). After the development of plate, spots were observed under UV light and fluorescence intensity of the sample spot was compared with the intensity of standard spot. By matching the intensity of sample with standard spot, concentration of aflatoxin was calculated with the formula:

$$\frac{S \times Y \times V}{X \times W}$$

Where, S= µl aflatoxin standard which matches the unknown

Y= concentration of AF B_1 standard (µg/kg)

V= µl of final dilution of sample extract

X= µl of sample extract spotted giving fluorescence intensity equal to S

W= wt. in gm of the sample contained in final extract[1].

RESULTS

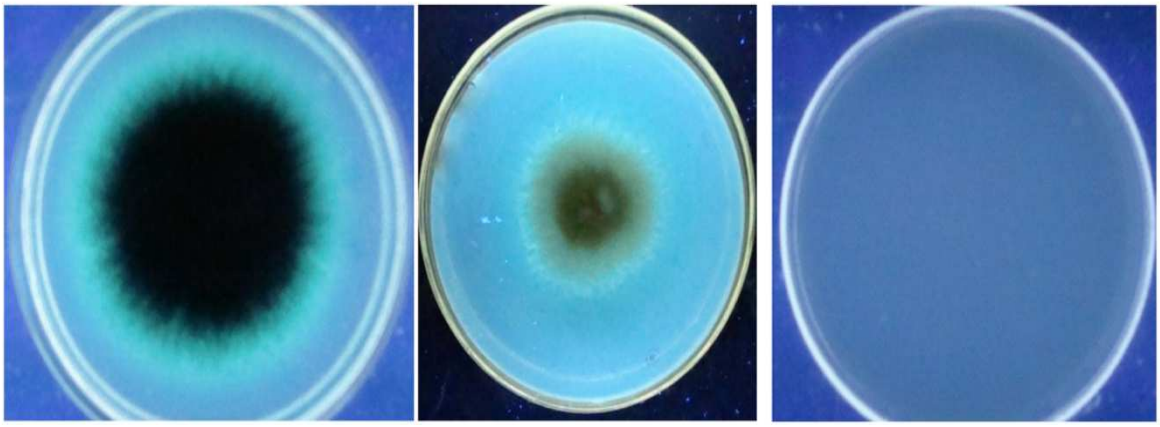
In the present study, an effort was made to isolate strains of *Aspergillus* species from rhizosphere of groundnut. From the samples collected, we identified ten *Aspergillus* species mainly *A. flavus, A. parasiticus, A. niger, A. repens, A. fumigatus, A. kanagawaensis, A. ochraceus, A. sydowi, A. terreus and A. nidulans.* Colony characteristics used for identification of *Aspergillus* isolates were shown in Table-I.

A total of 15 soil samples from the rhizosphere of groundnut were collected from eight different areas of Akola and Amravati District. Among the all the *Aspergillus* isolates from the rhizosphere of groundnut, it was found out that *A. fumigatus, A. niger, A. terreus* and *A. nidulans* was more frequent. All the collected soil sample was positive for *A. fumigatus and A. niger*. *A. nidulans* was also common along with *A. fumigatus*. The *Aspergillus* species was identified in lab and further confirmed by sending cultures to Indian type culture collection (ITCC), IARI, New Delhi.

Morphological characteristics used for identification of *Aspergillus* was shown in Table-II. Isolated *Aspergillus* species are tested for aflatoxin production which showed blue-green fluorescence on coconut agar medium. The species *A. flavus and A. parasiticus*, which are potent aflatoxin producer, shows the fluorescence on the reverse side of colony
within 2 days (photoplate-I).

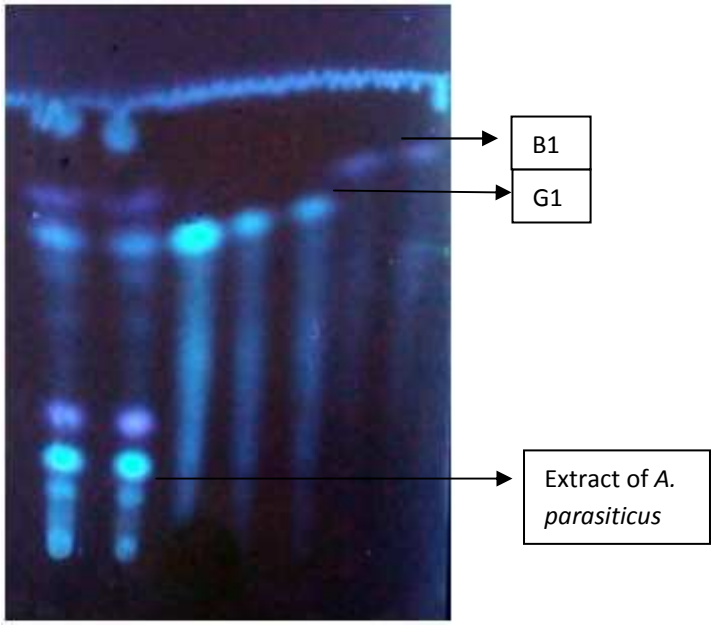
Toxigenicity of the isolates was quantified based on the intensity of the bands on TLC plates. Out of ten isolates of *Aspergillus* species isolated, it was found out that only extract of *A. flavus* and *A. parasiticus* showed the fluorescing spots on TLC plate at an Rf value of 0.45 (photoplate-II). A

similar Rf value was recorded for AFB_1 standard. Fluorescing spot with Rf 0.34 was identified from *A. parasiticus*. Rf value of this spot matched the Rf of the authentic standard of AFG_1.



(A. flavus) **(*A. parasiticus*)** **(control)**

Photoplate I: colonies of aflatoxin positive *A. flavus* and *A. parasiticus* on coconut agar medium under UV light showing characteristics fluorescence on agar.



Photoplate II: TLC plate showing the screening of AF B_1 and G_1

Table- 1: Macroscopic characteristics of *Aspergillus* species examined after 7 days of growth at 25° C on PDA, RBA, CYA and CzSA.

Characteristics	*Aspergillus* species	PDA	RBA	CYA	CzSA (10 days incubation)
Colony diameter (cm)	*A. flavus*	5.9	6.4	5.3	6.8
	A. parasiticus	6.8	6.2	6.6	3.6
	A. niger	6.1	4.6	5.4	3.5
	A. repens	6.9	5.1	6.2	5.0
	A. fumigatus	5.1	4.1	4.8	4.1
	A.kanagawaensis	5.7	4.9	5.3	3.4
	A. ochraceus	4.8	3.2	4.9	2.9
	A. sydowi	4.9	2.6	2.9	3.6
	A. terreus	3.6	4.7	3.6	3.5
	A.nidulans	4.8	5.1	3.8	4.3
Conidial color	*A. flavus*	Olive-lime green	Parrot green	Yellow-pale green	Yellow-dark yellow
	A. parasiticus	yellow- Dark green	Yellow-Deep green	Olive green	Parrot green-dark green
	A. niger	Carbon black	Deep brownish Black	Dark brown	Deep brownish – black
	A. repens	Light green	Deep brownish -green	Green	Dull green
	A. fumigatus	Blue-dull green	Gray to green	Blue green-gray blue	Dull blue-green
	A. kanagawaensis	Yellow-dull orange	Pinkish-cinnamon shade	Velvety pink-brown	Dull blue-green
	A. ochraceus	Light	Velvety white	Deep brown-	Whitish- pale

Characteristics	*Aspergillus* species	PDA	RBA	CYA	CzSA (10 days incubation)
		yellowish		yellow brown	yellow
	A. sydowi	Yellow- dull green	Dull green to blue green	Pale green	Blue-green
	A. terreus	Rosy yellow-brown	Dark yellow	Light yellow	Faint yellow-wood brown
	A.nidulans	Pale brown	Pinkish cinnamon		Dark green
conidial reverse	*A. flavus*	Yellowish	Yellow – brown	Pale yellow - brown	Colorless - pale yellow
	A. parasiticus	Whitish-yellowish	Grey-yellowish	Brown-orange	Cream-light drab
	A. niger	Colorless-pale yellow	Yellowish	White-yellowish	Colorless-pale yellow
	A. repens	Yellowish-brownish	Brown-dark brown in centre	Yellowish-brownish	Grayish yellow
	A. fumigatus	Yellow - green	Yellow brown	Yellow green	Colorless-varying in shade
	A. kanagawaensis	Dull orange	Slightly wrinkled olive	Yellow - orange	Dull orange
	A. ochraceus	Colorless-pale yellow	yellow- pale green	Pale yellow - orange	Yellowish-greenish brown
	A. sydowi	Colorless to pale brown	Light yellow	Pale brown	Brown-dark grey
	A. terreus	Light yellow-lemon	Dark yellow to light orange	brownish	Dull brown
	A.nidulans	pinkish	Pink to brown	brownish	Purplish red-dark in age

Table- 2: Microscopic characteristics used for the identification of *Aspergillus* isolates

Fungus	microscopic features			
Aspergillus species	Color of conidial head	Vesicle serration	Conidial head	Conidia
A. flavus	Yellow when young, becoming dark yellow-green in age	uniseriate	Radiate, splitting into poorly defined column	Globose to subglobose
A. parasiticus	Parrot green to dull yellow green	Uniseriate	Globose	Globose to subglobose
A. niger	Large and black	Globose, thick walled, longer	Globose then radiate in well defined column	Globose to subglobose
A. repens	Orange-yellow	hemispherical	Radiate, split into columns with chain of conidia	Subglobose, globose
A. fumigatus	Light green to dull blue green	Uniseriate pyriform	Columnar, compact, densely crowded	Globose to subglobose, green in mass
A. kanagawaensis	Pinkish cinnamon shade	Slightly flattened	Loosely radiate with chain of conidia	Globose, slightly irregular from fragment
A. ochraceus	Light yellow to brown	Globose, thin walled	Globose, adhering compact columns	Globose to subglobose
A. sydowi	Blue-green	Globose to hemispherical	Globose to hemispherical	Globose to subglobose
A. terreus	Cinnamon-brown	hemispherical	Columnar, compact with uniform diam.	Globose to subglobose, smooth walled
A. nidulans	Dark green to pinkish cinnamon	hemispherical	Slightly larger than others	Globose to subglobose, green mass

Several defined and semi-synthetic culture medium have been designed and employed in the production of aflatoxins by *A. flavus* and *A. parasiticus* in surface culture fermentations and the data on aflatoxins concentrations and mycelial weight are presented in Table -3.

Table- 3: A comparative study on growth and aflatoxin B_1 production by *A. flavus* and *A. parasiticus* on variable liquid culture media.

Liquid Culture medium	*A. flavus*		*A. parasiticus*	
	Mycelial dry weight (gm/50ml)	Aflatoxin B_1 (µg/l)	Mycelial weight (gm/ml)	Aflatoxin B_1 (µg/l)
Richard broth	1.48	966.66	0.96	266.66
SMKY broth	0.90	933.33	0.67	233.33
Czapek-Dox broth	0.28	66.66	0.30	13.13
Czapek-Dox broth (8.6% glucose + 0.4% citric acid)	0.92	55.55	0.58	27.77
Yeast extract (2%+ sucrose 10%) broth	2.03	55.55	3.2	11.11
Yeast extract (2%+ sucrose 20%) broth	2.44	166.66	1.28	222.22

It can be seen from the results that among the medium used Richard broth were found to be best medium as compare to SMKY as better medium for aflatoxin production by both *A. flavus* and *A. parasiticus*. The Richard broth were able to produce the aflatoxin at maximum level of 966.66 – 266.66ug/l and SMKY broth 933.33 – 233.33 ug/l) for both species which further indicated by high fluorescence on TLC plates. It has been further confirm that liquid medium provides the intimate mixing of nutrients with the fermenting organism, utilization of nutrients, production and quantitation of aflatoxin was found to be faster and easier in Richard and SMKY liquid medium as compare to solid medium and other use medium (data not shown). It is interesting to note that other eight isolates associated with ground nut soil does not show any florescence on coconut agar medium as well as they do not exhibit any kind of fluorescence on TLC plates.

DISCUSSION

In this study, ten standard *Aspergillus* species were identified successfully. We demonstrate that use of four differential media including; PDA, RBA, CYA and CzSA was simple and reliable methods for identification of *Aspergillus* species. Although same studies with similar design was reported, but this is useful to study the fungal infection, damage to the seeds of groundnut. Use of different sporulation media for isolation of *Aspergillus* species may speed up the growth rate and in the production of conidia[19].

The above results shows that from the ten species of genus *Aspergillus, A. fumigatus, A. niger* and *A. nidulans* was most common strains isolated from rhizosphere of groundnut. Their common occurrence could also be due to their high sporulating nature and this is also coupled with their ability to grow well on laboratory media[20, 7]. The rare isolated species were *A. kanagawaensis, A. repens, A. terreus* and *A. ochraceus* reported in the study.

In the culture and identification method, *A. fumigates* was most rapid grower. The typical gray-green colonies develop within 24 - 48h on both PDA and RBA media. On CYA and CzSA media growth rate was much slow. Other Aspergilli isolated i.e. *A. flavus, A. parasiticus, A. niger, A. repens, A. kanagawaensis* and *A. terreus* have growth rate near about similar to that of *A. fumigatus* when colonies were observed on PDA and RBA media, after incubation for 5 days at 25°C. Growth of *A. ochraceus* was slightly slow. The use of differential media helps to accelerate growth rate and the production of conidia[5, 23].

The use of coconut agar medium has greatly lowered the cost of rapid aflatoxin screening from fungi. As the fungus grow more rapidly excellent results were obtained in 2-4 days than 7-8 days as done by (16) and (4), because on the 7th day screening becomes difficult to evaluate as the mycelial growth reached the margin of the plate and obscured the blue fluorescence. It was found that rapid screening of aflatoxin production on coconut agar medium was easy as the media was easy to prepare and detect. Detection of aflatoxin production by the presence of fluorescence is rapid and specific method and also the presence of orange yellow pigmentation prior to fluorescence can be act as presumptive screening method for aflatoxin production without the use of UV lamp [11].

The use of various liquid medium along with its modification on the growth and aflatoxin production by *A. flavus* and *A. parasiticus* strains investigated[13, 3]. Unlike many secondary metabolites, aflatoxin biosynthesis is induced by the presence of simple carbohydrates, especially glucose, sucrose, fructose and maltose, but not by carbohydrates sources such as peptone, lactose, sorbose and oleic acid[17]. Glucose analogous that are not readily metabolized failed to induce aflatoxins. It was already shown that sucrose concentration of 3, 10, 20 and 30% supported both growth and sporulation, but that the 10% to 20% sucrose concentration was most conductive to aflatoxin biosynthesis. But in present study the 50% and 20% of sucrose concentration in Richard medium and SMKY medium supports the fact and gives higher quantity of aflatoxin concentration which is in opposite of higher growth found in Yeast extract medium with modifications.

In the present study the pH of the medium was kept at 5, as according to Ehrlich *et al*. (2005) production of aflatoxins by *A. flavus* and *A. parasiticus* is known to occur only at acidic pH. A slight fluctuation on pH of the medium had no effect on aflatoxin production or growth.

Salts like sodium chloride and potassium chloride enhance aflatoxin production by toxigenic fungus in lower concentration, but decrease at higher concentration (8-10% sodium chloride for *A. flavus* and *A. parasiticus*[10]. When nitrogen was supplied in the form of the various inorganic salts in different media used for aflatoxin production, it was found out that 1% KNO_3 was the best salt use in Richard medium[6] and SMKY[12, 21] promotes aflatoxin production as compare to others $NaN0_3$ in Czapek Dox and organic source as yeast.

The present study gives simple way of isolation, characterization of soil mycoflora associated with groundnut rhizosphere and also the rapid detection and characterization of type of aflatoxin produced by the selected strains of *A. flavus* and *A. parasiticus.*

ACKNOWLEDGMENT

The authors express their sincere thanks to UGC, New Delhi for providing funds under Research award scheme 2009-11 and Rajiv Gandhi National fellowship. Also thanks are extending to T. Prameela Devi, ITCC, IARI, New Delhi for confirmation of cultures.

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