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Full Length Research Paper

Characterization of *Aspergillus* species associated with commercially stored triphala powder

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About 82 triphala powder samples were analyzed for the association of different fungi. Results reveal the predominance of *Aspergillus* as the major genera with six predominant species namely, *A. niger, A. flavus, A. fumigatus, A. terreus, A. nidulans* and *A. amstelodami*. Therefore, these six isolated *Aspergillus* species were characterized morphologically, microscopically and molecularly. In addition, an attempt was made to characterize the frequently occurring aflatoxigenic and non toxic *Aspergillus* species at molecular level along with their identification. Morphological variability was detected among isolates in regard to colony morphology, conidia colouration, exudates and reverse, colony texture and growth rate. Microscopic characteristics for the identification were conidial heads, stipe, colour and length of vesicles, shape and seriation, metulae covering, conidia size and shape. For molecular characterization, internal transcribed spacer (ITS)-4, a universal fungal primer was utilized. Differences in band patterns and number of bands obtained after polymerase chain reaction (**PCR) amplification clearly differentiates between the *Aspergillus* species. Results also reveal that only *A. flavus* showed amplification with all the three aflatoxigenic primers apa-2, ver-1 and omt-1, which means that only *A. flavus* was identified as aflatoxigenic and other *Aspergillus* species as non-toxigenic after PCR analysis. Hence, morphological, microscopic and molecular methods are important for the complete identification of important *Aspergillus* species and other fungi isolated from stored commodities.**

Key words: *Aspergillus*, triphala, identification, macroscopic and microscopic characteristics, molecular methods.

INTRODUCTION

Ayurveda is one of the oldest and richest practices in Indian systems of medicine, used for curing various ailments. Among the different ayurvedic formulations, triphala powder is one of the worldwide popular Indian formulation. It is an equiproportational (1:1:1) mixture of fruit powders of *Emblica officinalis* (Gaertn.) (Amla), *Terminalia bellirica* (Gaertn.) Roxb. (Baheda), and *Terminalia chebula* (Retz.) (Harada), used in about every home, to cure various problems like constipation or indigestion, dyspepsia, anaemia, hyperlipidaemia, skin diseases, excessive heat and irritation of eyes. In addition to promoting regularity processes of digestion, these fruits are traditionally held to support blood purification, bile secretion, and to maintain the health of gastrointestinal tract lining (Kulkami, 1995; Juss, 1997). A significant factor that affects triphala powders quality with regard to health and safety is the occurrence of mycotoxins and their producers, microscopic fungi, during various steps of their preparation. Since, triphala churn is of plant origin that is, prepared from dried fruit, it can also become contaminated by *Aspergillus* strains during growth (while the fruits are on tree), after harvesting, processing (when fruits are dried) and during storage. In addition, the different biotic factors like temperature, moisture, and relative humidity, as well as of processing area and methods used during processing to pre and post-harvest practices, are also the main cause and sources for fungal association (Truckesses and Scott, 2008). Different *Aspergillus* species are reported to be associated as major fungi with plant based drugs during

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different steps of their preparation (Mandeel, 2005; Gautam and Bhadauria, 2008, 2010a, c; Sareen et al., 2010).

Aspergillus species are known to produce a broad spectrum of mycotoxins including aflatoxins, sterigmatocystin and ochratoxins, which are causative agents of several carcinogenic, hepatogenic, nephrogenic, and immunosuppressive effects (Muntanola, 1987; Singh et al., 1990; Rai and Mehrotra, 2005; Moss, 2006; Dragan et al., 2010; Gautam and Bhadauria, 2010b). Therefore, the correct identification of associated *Aspergillus* species with stored herbal drugs is necessary, in order to avoid the ailments caused and make an early recognition of invasive fungal infection easier to reducing the mortality associated with disseminated disease. The classical methods performed, by examining several morphological traits observed on fungal cultures, grown on different media and microscopic characteristics are the most widely used tools for fungal identification. However, these procedures are time consuming, require important mycological knowledge and are inaccurate because of intra and inter specific morphological divergences (Klich and Pitt, 1988). This promoted towards molecular techniques like randomly amplified polymorphic DNA (RAPD) in diagnosis of fungi to identify fungi more accurately.

The objective of this present study was to characterize the different *Aspergillus* species, employing morphological, microscopic and molecular methods. The use of molecular techniques based on the RAPD analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) have been useful for the identification and classification taxonomy of this filamentous fungal genera. In addition, an attempt was made to characterize the frequently occurring *Aspergillus* species for their mycotoxic and non toxic nature at molecular level.

MATERIALS AND METHODS

Sample collection

A total of 82 triphala powder samples of different brands were randomly collected during the year 2007 to 2009, transported to laboratory and stored for further analysis. The moisture content of all the collected samples was determined by oven drying at 80°C until their weights remains constant (Essono et al., 2007) and the difference in weight was calculated as:

$$MC = [(Wi - Wf)/Wi] \times 100$$

Where, MC is the moisture content; Wi is the initial weight and Wf is the final weight

Mycoflora isolation

One gram of each powdered sample was mixed aseptically in 9 ml of sterile distilled water and shaken vigorously. Appropriate tenfold serial dilution was made and 0.1 ml of the dilution was transferred aseptically to sterilized Petri plates, containing growth media. For mycobiota analysis, freshly prepared potato dextrose agar (200 g peeled potatos, 20 g agar and 15 g agar in 1,000 ml of distilled water) and czapek dox agar medium (2 g $NaNO_2$, 1 g K_2HPO_4, 0.5 g $MgCl_2$, 0.5 g KCl, 0.01 g $FeSO_4$, 30 g sucrose and 15 g agar in 1000 ml distilled water) were used. Triplicate of each sample were incubated at 25 ± 2°C, for 7 days and examined visually as well as under a compound light microscope daily for preliminary identification of fungal genera. The identified genera were then sub-cultured on suitable agar plates for species identification.

Macroscopic and microscopic identification

Identification of fungal species was done on the basis of cultural and morphological characteristics. Macroscopic features like colony colour, texture and margins, as well as microscopic such as size of conidia and conidiophores and their arrangements were examined for species differentiation (Raper and Fennell, 1965; Gilman, 2001). Most frequently occurring *Aspergillus* species were further validated by culture, at Indian Agricultural Research Institute (IARI), New Delhi, India.

Molecular methods

Pure culture of most frequently occurring fungal isolates isolated form triphala churn and its ingredients were maintained. For DNA isolation, the cultures were grown in potato dextrose broth (PDA; pH 5.5) for 7 days at 28 ± 1°C. Mycelia were filtered through filter paper (Whatman no. 1) and DNA was extracted, using the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). The mycelium was ground into fine powder with glass beads, transferred to DNA extraction buffer (0.1 M Tris, 1.5 M NaCl, 0.01 M EDTA) and kept at 65°C, for 1 h, with occasional stirring. Equal volumes of chloroform, Isoamyl alcohols (24:1), were added to all tubes, followed by centrifugation. The upper aqueous phase obtained was precipitated with 0.6th volume of ice-cold isopropanol and 0.1th volume of 3 M sodium acetate (pH 5.2) and again centrifuged. The pellet obtained was washed with 70% ethanol and dried at room temperature. Finally, obtained DNA pellet was dissolved in TE buffer and stored at -20°C. The fungus-specific universal primer ITS-4, synthesized by Merck Specialities Private Limited, India, were used to amplify genes encoding the ITS region (Tarai et al., 2006). In addition of universal primers, three mycotoxin specific primers, apa-2, ver-1 and omt-1 (Shapira et al., 1996; Konietzny and Geriner, 2003) were also used to differentiate between mycotoxic and non toxic *Aspergillus* isolates (Table 1). All the PCR reagents like Taq polymerase, 200 µM dNTP (dATP, dCTP, dGTP, dTTP), and reaction buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) used were of Merck Specialities Private Limited, India.

Concentrations of DNA template, primer and deoxynucleotide triphosphates (dNTPs), and the optimum annealing temperature were standardized for each primer in preliminary trials. PCR was performed in a total reaction volume of 25 µL which consisted of 2 µL of target DNA solution, 3 µL (6 µL in ITS-4) of each (forward and reverse) of the primers and 17 µL of milliQ water. The mixture was spinned before subjected to PCR. The amplified PCR products were electrophoresed on a 1% agarose gel in tris-borate-EDTA (TBE buffer), visualized by staining with ethidium bromide and photographed, using a gel documentation system (Uvitec, U K).

Statistical analysis

The analysis of data was performed with Microsoft excel 2007(Windows XP) for mean and standard deviation. Descriptive

Table 1. Primers sequences used for amplification of ITS-4 region and aflatoxin synthetic genes.

Primer	Sequence 5'-3'	PCR product size (bp)
ITS-4 (Universal primer)	5´ TCC TCC GCT TAT TGA TAT GC-3´	
Primer	**Sequence 5'-3'**	
apa-2	5´-TATCTCCCCCCGGGCATCTCCCGG3´ 5´-CCGTCAGACAGCCACTGGACACGG-3´	1032
Ver-1	5´-TGTCGGATAATCAGCGTTTAGATGGC-3´ 5´-CGAAAACGCCCACCATCCACCCCAATG-3´	895
omt-1	5´-GTGGACGGACCTAGTCCGACATCAC-3´ 5´-GTCGGCGCCACGCACTGGGTTGGGG-3´	596

Table 2. Percent relative frequency and density of isolated *Aspergillus* species from triphala powder samples.

Fungal species	Relative frequency		Relative density	
	X = 82	(%)	N	(%)
Aspergillus niger	56	68.29	745	49.76
A. flavus	35	42.68	247	16.49
A. amstelodami	15	19.51	246	16.43
A. fumigatus	37	45.12	143	9.55
A. terreus	10	12.19	34	2.27
A. nidulans	09	10.97	82	5.47

X **=** No. of samples, N = No. of fungal isolates.

analysis of percent relative density and frequency for *Aspergillus* isolates were performed on the collected data.

RESULTS

The variation in percent moisture content in triphala powders was recorded in the range of 4.16% to 10.29%. A total of 63.41% samples were found contaminated with different fungal species, with *Aspergillus* as the major genera. Among *Aspergillus*, six species namely, *A. niger, A. flavus, A. fumigatus, A. terreus, A. nidulans* and *A. amstelodami* were found associated with the samples analysed. Relative density and frequency of the different *Aspergillus* spp. were presented in Table 2. Morphological microscopic and molecular characteristics were studied for identification of all isolates, along with available literature.

Among the isolated *Aspergillus* species, highest frequency was observed in *A. niger* (68.29%), followed by *A. fumigatus* (45.12%) and *A. flavus* (42.68%), while it was 19.51% in *A. amstelodami,* 12.19% in *A. terreus* and 10.97% in *A. nidulans,* respectively. Similarly, the highest relative density was shown by *A. niger* (49.76%), while moderate was by *A. flavus* (16.49%) and *A. amstelodami* (16.43%). The lowest relative density was recorded in case of *A. fumigatus* (9.55%), *A. terreus* (2.27%) and *A. nidulans* (5.47%).

Macroscopic and microscopic analysis

Analysis of different *Aspergillus* species isolated from triphala powder samples for morphological and cultural characterstics showed that there was variation in the colony colour, margins, texture and colony reverse colours (Figures 1a, b and 2). The morphological characteristics of *Asplergillus* isolated which are *A. niger, A. flavus, A. fumigatus, A. terreus, A. nidulans* and *A. amstelodami* are depicted in Table 3. After macroscopic, the isolated *Aspergilus* isolates were examined for their microscopic characters and are presented in Figure 3 and Table 3.

Molecular analysis

Analysis of the ITS regions

Amplification of the ITS-4 regions from the six clinically relevant *Aspergillus* strains generated PCR products ranging in size from 300 to 1185 bp (Figure 4), a total of 14 bands, 1 to 6 in number, with 0.3 to 1.185 kb size,

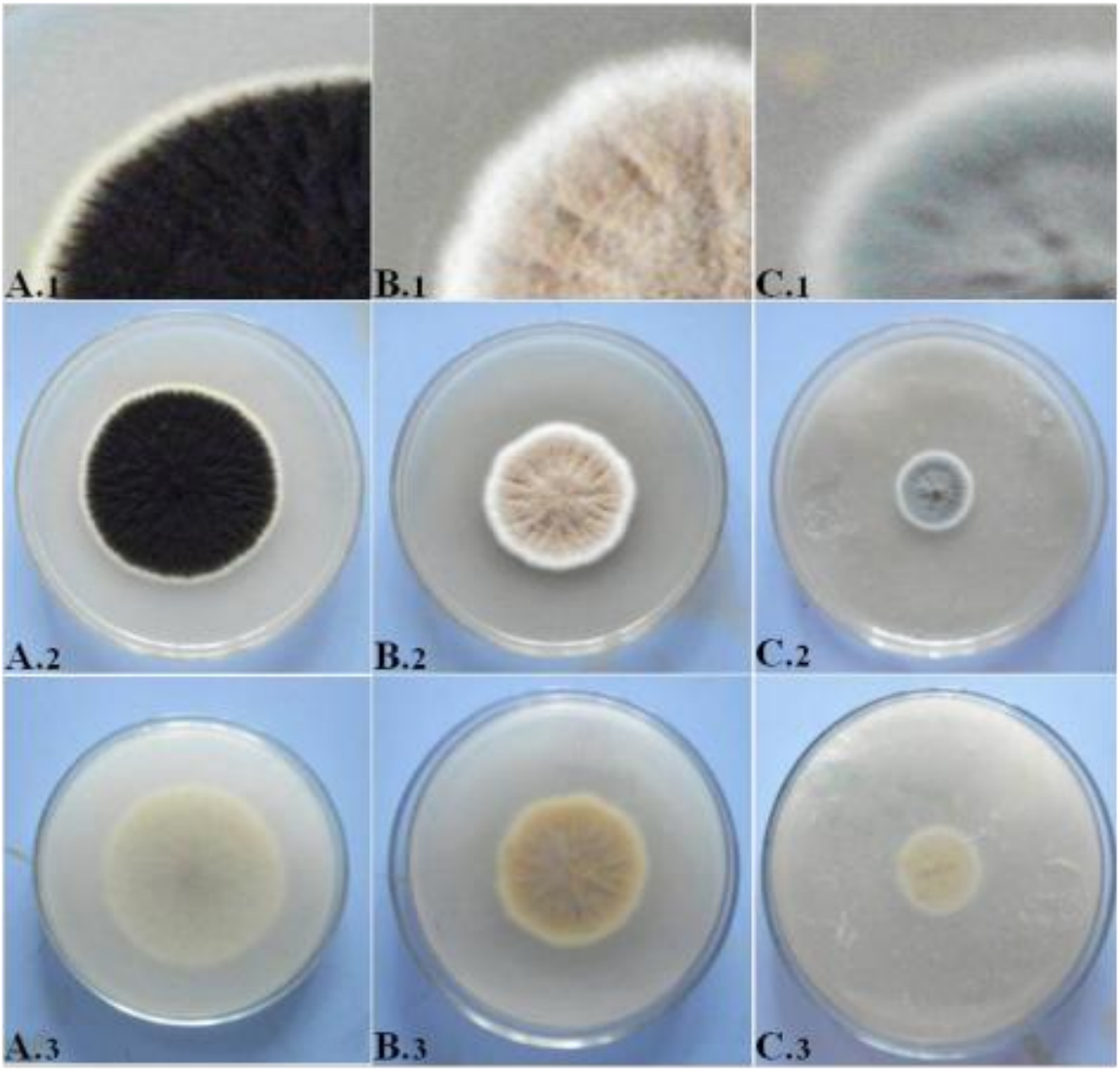

Figure 1a. Morphological characterization of different *Aspergillus* species on czapek dox agar: A: *Aspergillus niger*; B: *A. terreus* and; C: *A. fumigatus.* (1 = margin; 2 = surface colour and; 3 = reverse side of colony).

showing variability in number and size of fragments of different *Aspergillus* isolates. Six bands, ranging between 300 to 900 bp in size, were detected in *A. fumigatus*. Two bands were recorded in three fungal isolates namely, *A. niger* (300 and 400 bp), *A. terreus* (900 and 1185 bp), *A. amstelodami* (600 and 900 bp). Only single band was observed in *A. flavus* (900 bp) and *A. nidulans* (300 bp).

PCR specificity with aflatoxigenic primers apa-2, ver-1 and omt-1

All the six *Aspergillus* isolates were tested for their aflatoxigenic nature with omt-1, ver-1 and apa-2 gene specific primers. As expected, amplification products of 895 bp, 596 bp and 1032 bp, regardless of primer set used were obtained only from *A. flavus.* No such amplified products were not detected in remaining isolates of test organisms, which means only *A. flavus* was identified as aflatoxigenic and *A. niger, A. terreus, A. fumigatus, A. amstelodami* and *A. nidulans* as non-toxigenic strains during PCR analysis (Figure 5).

DISCUSSION

Contamination of stored triphala powder samples, with high frequency of different *Aspergillus* species and production of related mycotoxins poses a risk for consumer's health. *Aspergillus* is also recovered as major fungal genera contaminating stored herbal drugs (Hitokoto et al., 1978; Aziz et al., 1998; Arab et al., 1999; Elshafie et al., 1999; Mandeel, 2005; Gautam and

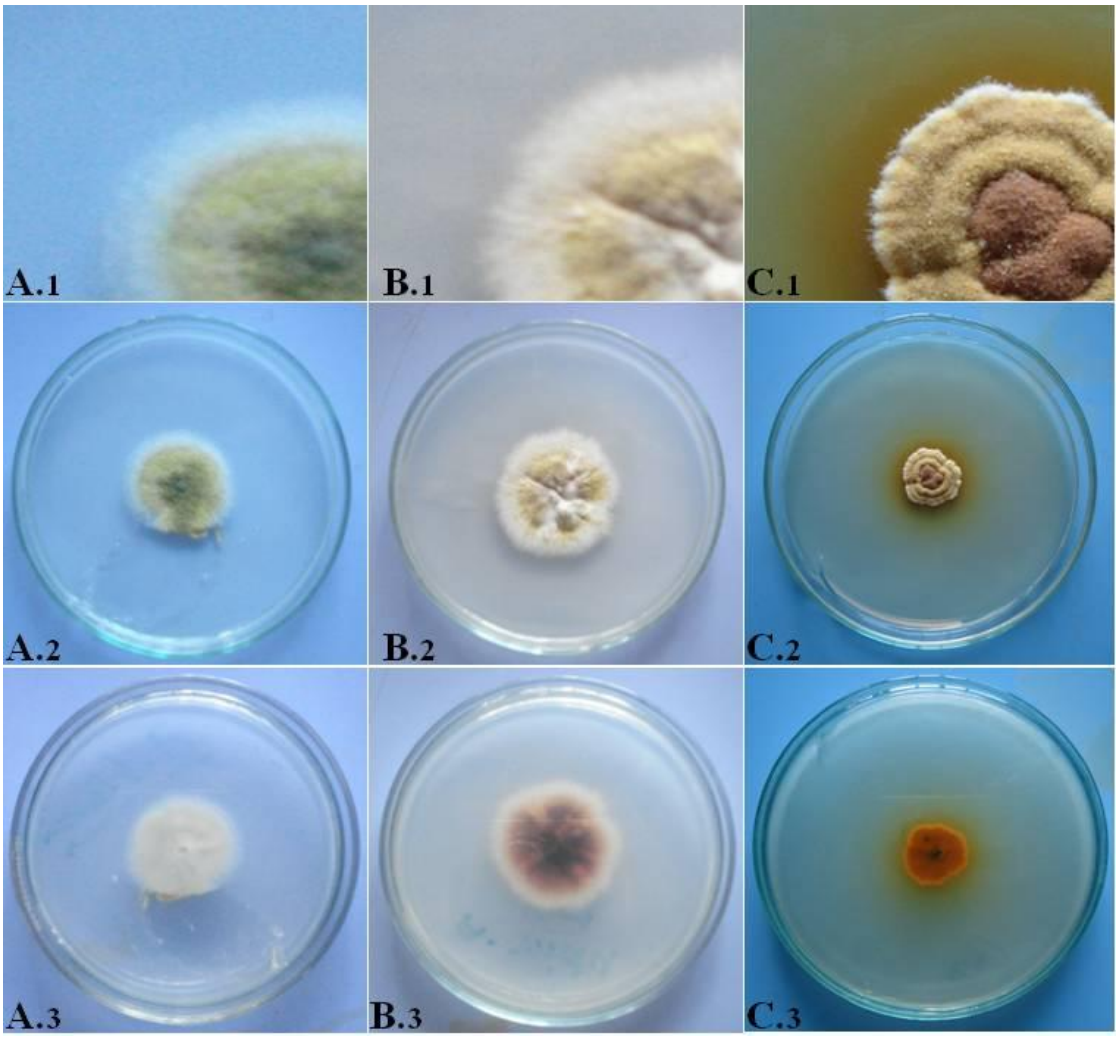

Figure 1b. Morphological characterization of different *Aspergillus* species on czapek dox agar: A: *Aspergillus flavus*; B: *A. nidulans* and; C: *A. amstelodami.* (1 = margin; 2 = surface colour and; 3 = reverse side of colony).

Bhadauria, 2008, 2010a, c; Sareen et al., 2010). *Aspergillus flavus* is detected in great frequency from the triphala powder samples analysed in the present study which poses a risk to the consumers because of its ability to produce aflatoxins, the potent carcinogens (Diener et al., 1987).

Detection of *A. niger* and *A. fumigauts* is also of great concern because both are dangerous allergen, associated with apergillosis (Abraca et al., 1994; Schuster et al., 2002; Noonimabc et al., 2009; Edwin et al., 2010; Gautam et al., 2011). This highlights the importance of correct identification and taxonomical differentiation between different species of *Aspergillus*.

The taxonomy of *Aspergillus* has always been complex due to its great number of species (nearly 250), which have very few differences. The identification of different *Aspergillus* species, on the basis of their morphological characters (example, colony colours, reverse side and margins) is one of the oldest and most adopted methods. Some of the species of *Aspergillus* have the same morphological features which make it difficult to distinguish between them.

This shows that morphological characters are not enough for fungal identification and it renders the need of microscopic features along with the morphological. Same colony colours of *A. niger* and *luchuensis*; *A. flavus* and *oryzae; A. anstelodani* and *parasiticus* supports the requirement of microscopic characters, along with the macroscopic for correct identification and classification.

The fungal identification on the basis of their macroscopic and microscopic features become classical method and still most widely and commonly used tool in the field of fungal taxonomy.

This fact helps researchers in understanding fungal ecology, diversity and consequently their exploration for industry.

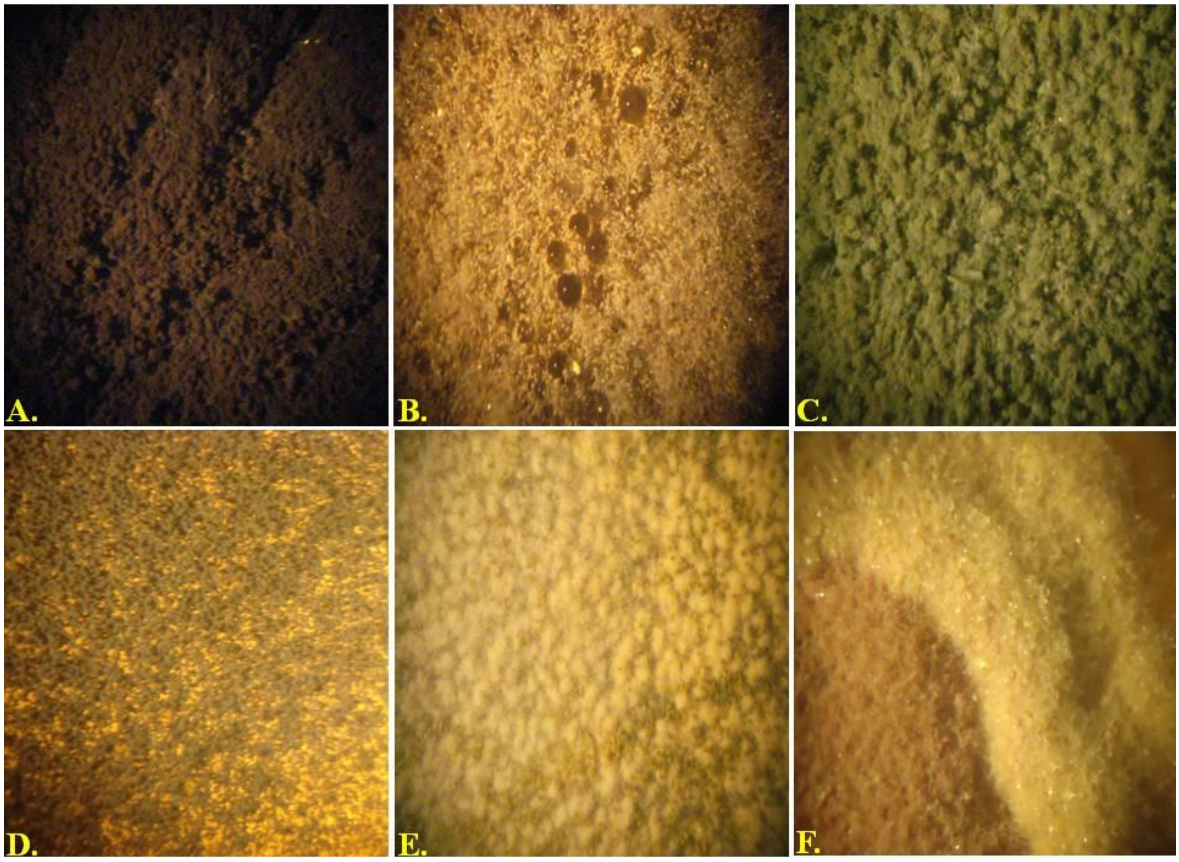

Figure 2. Colony texture of different *Aspergillus* species under stereo microscope: A: *Aspergillus niger*; B: *A. terreus*; C: *A. flavus*; D: *A. fumigatus;* E: *A. nidulans* and; F: *A. amstelodami.*

Although, the conventional morphological and microscopic characterization are most popular classical methods but, it is believed that these are time consuming and are not sufficient to characterize the different fungal species, due to their intra and inter specific morphological divergences (Klich and Pitt, 1988; Samson et al., 2004). Molecular characterization on the other hand, is a rapid and quick procedure which requires minimal handling and able to distinguish even morphologically, similar fungal species. Therefore, molecular characterization has also been carried out in the present investigation, to identify the frequently isolated six species of *Aspergillus* with the help of pan fungal universal primer ITS-4. Variation numbers of bands and their pattern in different *Aspergillus* species was observed, which became helpful to distinguish them. Several such studies on the use of PCR technology for the detection and diagnosis of fungi, by using the internal transcribed spacer (ITS) have already been published (Henson and French, 1993; Marek et al., 2003; Haughland et al., 2004; Druzhinina et al., 2005**)** which also support the present study. With the help of RAPD-PCR, Khan et al. (2007) studied diversity in *Aspergillus niger* isolates collected from pigeon pea field in Aligarh region. After RAPD analysis, a set of different band patterns of amplified DNA with PCR were observed for different genome. Similar studies were also carried out recently by Godet and Munaut (2010) in differentiating *Aspergillus flavus, A. parasiticus, A. tamarii* and *A. nomius* by PCR-RAPD markers. Likewise, Leema et al. (2010) confirmed the *A. flavus* by molecular methods that is, amplification of the internal transcribed spacer 2 (ITS 2) regions.

Several molecular techniques have been tested to classify different *Aspergillus* species like random amplification of polymorphic DNA (RAPD) (Yuan et al., 1995), the internal transcribed spacer (ITS) region (Kumeda and Asao, 1996; Henry et al., 2000; Kumeda and Asao, 2001; Rigo et al., 2002) and the aflatoxin gene cluster (Chang et al., 1995; Watson et al., 1999; Tominaga et al., 2006).

Out of six *Asperigillus* isolates subjected to PCR analysis with aflatoxigenic primers apa-2, ver-1 and omt-1, only *A. flavus* showed the amplification, indicating their potential aflatoxin producing ability. The use of apa-2, ver-1 and omt-1 as probe, can discriminate between aflatoxin producing toxigenic and non toxigenic fungi. Similar observations by using ver-1, omt-1 and aflR (Shapira et al., 1996) and nor-1, ver-1 and omt-1

Table 3. *Aspergillus* species showing morphological and microscopic variation. *Aspergillus niger* (#ITCC 7414.09); *A. flavus* (#ITCC 7413.09); *A. fumigatus* (#ITCC 7408.09); *A. terreus* (#ITCC 7406.09); *A. amstelodami* (# 7411.09) and; *A. nidulans.*

Characteristics	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus terreus*	*Aspergillus nidulans*	*Aspergillus amstelodami*
Morphological characteristics of the colony						
Surface colour	Dark brown to black	Yellow/greyish green	Green to darkgreen, becoming black with age	Pinkish cinnamon to deeper with age	Dark cress green	Dark green
Margins	Entire	Entire	Entire	Entire	Entire	Entire
Reverse side	Without colour	Colourless to yellow	Colourless to yellow	Pale to bright yellow to deep brown	Purplish red, brownish dark with age	Brownish red
Elevations	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate
Growth	Rapid	Moderate to rapid	Rapid	Moderate to rapid	Slow to moderate	Very slow
Microscopic characteristics						
Hyphae	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate	Branched septate
Conidiophore						
a) Length	200 to 400µm	600 to 800 µm	250 to 300 µm	150 to 250 µm	60 to 150 µm	200 to 350 µm
b) Diameter	7 to 10 µm	15 to 20 µm	2 to 8 µm	5 to 8 µm	8 to 12 µm	7 to 10 µm
c) Vesicle	Globose	Globose to subglobose	Dome shaped	Globose	hemispherical	globose
Conidia	30 to 75 µm	20 to 45 µm	20 to 30 µm	2 to 3µm	8 to 12 µm	3.9 to 4.2 µm
a) Heads	Blackish brown	Yellow/greyish green	Blue green	Creemish	Cinnamon brown	Yellowish green
b) Diameter	2.5 to 4 µm	2 to 6 µm	2.5 to 3 µm	2 to 5 µm	3 to 3.5 µm	4 to 7 µm
c)Ornametation	Exine spiny	Almost smooth	roughened	Smooth	Almost smooth	roughened
Phialides	Two series (Biseriate) covering entire vesicle	Two series (Biseriate) covering nearly entire vesicle	Single series (Uniseriate) covering only upper portion of vesicle	Two series (Biseriate) covering only upper portion of vesicle	Two series (Biseriate) covering only upper portion of vesicle	Single series (Uniseriate) covering nearly entire vesicle
a) Primary	20 to 30 µ	7 to 10 µm	6 to 8 µm	7 to 9 µm	5 to 6 µm	40 to 60 µm
b) Secondary	40 to 60 µ	7 to 10 µm	Absent	5 to 7 µm	5 to 6 µm	Absent
Fruiting body	Cleistothecia present	Cleistothecia present	Cleistothecia present	Cleistothecia absent	Cleistothecia present Hulle cells present	Cleistothecia present

(#ITCC = Indian Type Culture Collection, New Delhi, India).

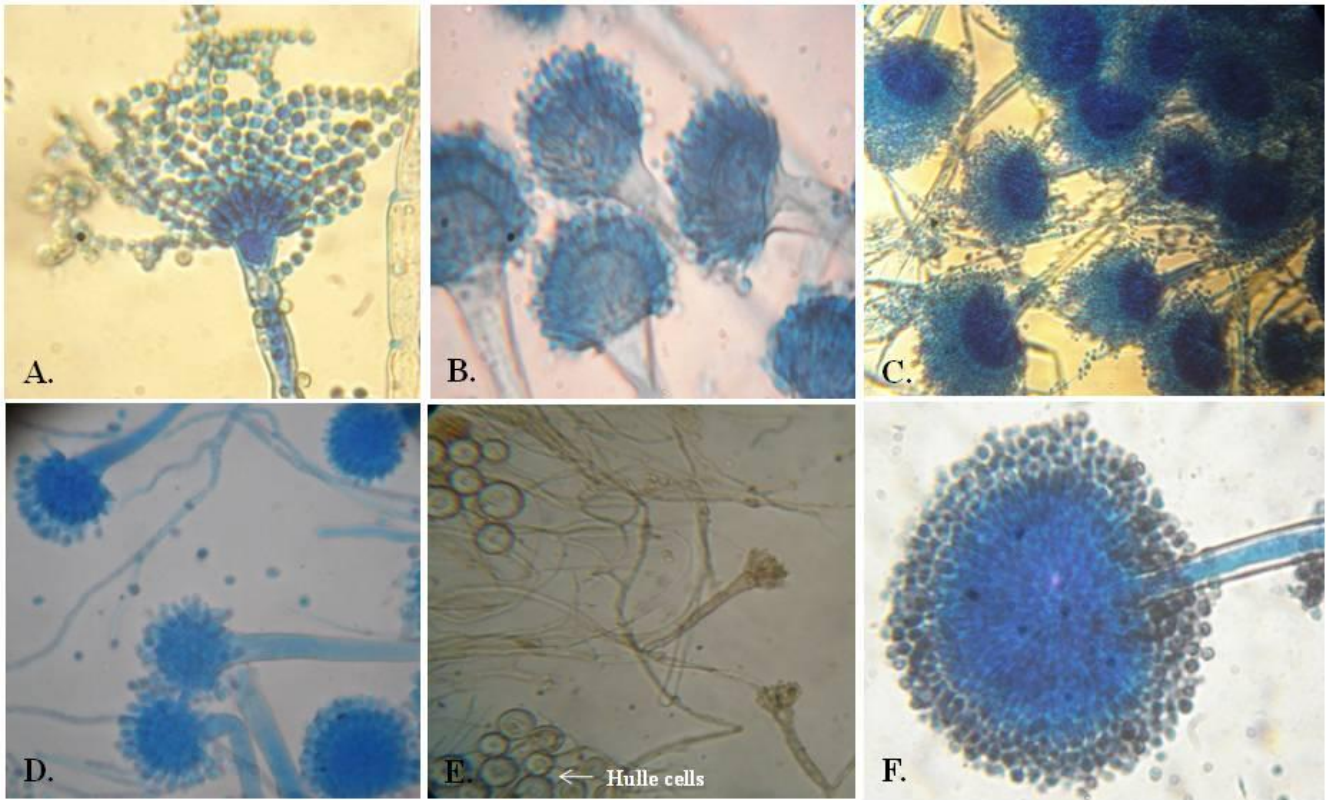

Figure 3. Microscopic characters of *Aspergillus* isolates: (A) *A. flavus;* (B) *A. fumigatus;* (C) *A. terreus;* (D) *A. amstelodami;* (E) *A. nidulans* with hulle cells and; (F) *A. niger.*

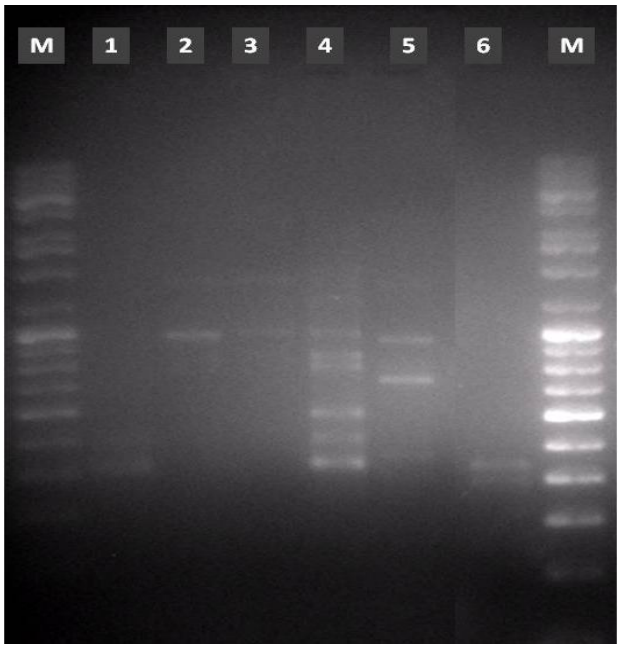

Figure 4. Band pattern of different *Aspergillus* isolates resulted from PCR reactions primed by ITS-4 universal fungal primer. Lane M = marker; lane 1 = *A. niger*; lane 2 = *A. flavus*; lane 3 = *A. terreus*; lane 4 = *A. fumigatus*; lane 5 = *A. amstelodam*; lane 6 = *A. nidulans* and; M=marker.

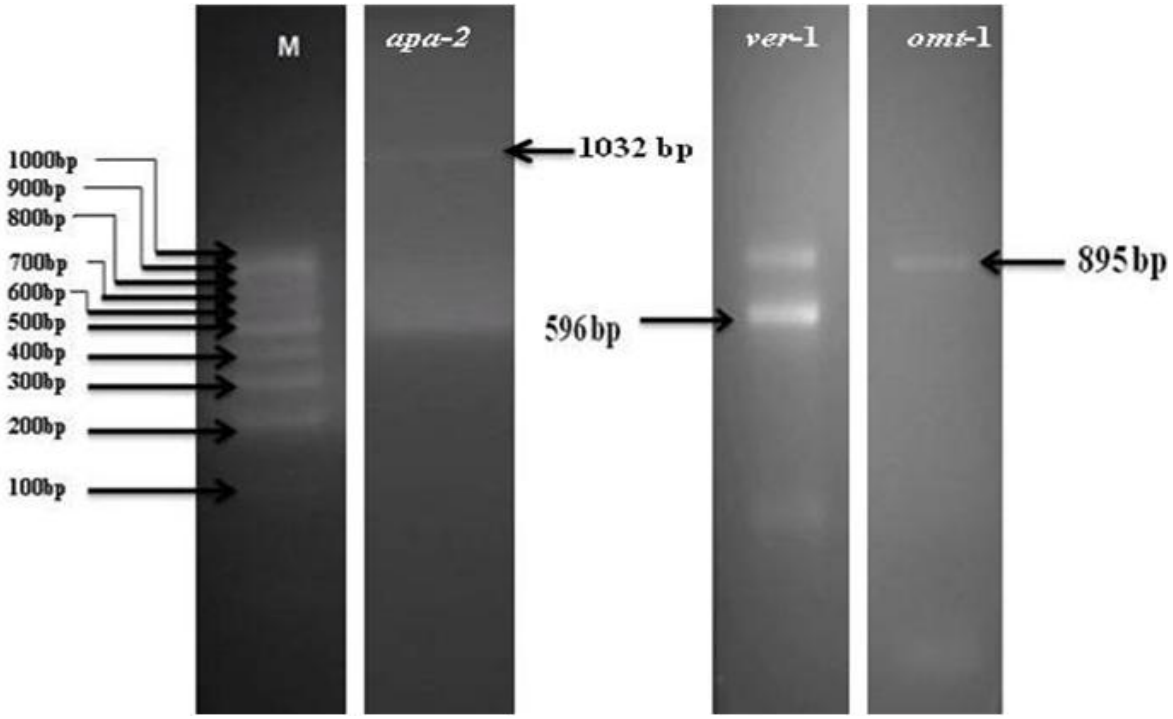

Figure 5. PCR amplification with genomic DNA of *Aspergillus flavus* species primed by apa-2, ver-1 and omt-1.

(Geisen, 1996) as RAPD genetic markers have also been reported to differentiate, aflatoxigenic and non aflatoxigenic strains of *A. flavus* and *A. parasiticus*. Maheshwar et al. (2009) have also reported the association of toxigenic endophytic *Fusarium verticillioides* in maize by PCR analysis.

Conclusion

The importance of herbal drugs like triphala powder for consumer's health, and the correct identification of fungal contaminants is very important at early stages, to avoid their health hazardous risk. Macroscopic and microscopic methods are sufficient enough for identification, and the molecular studies makes it more accurate and helpful in selecting protection measures. None of the methods is able to solve problems of identification singly. In our view, morphological, microscopic and molecular methods are equally important for the complete identification and confirmation of important *Aspergillus* spp. and other fungi.

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