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Detection of black Aspergilli and ochratoxin a contamination on grapes in Tamil Nadu, India

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Abstract
Grapes is the one of the most important fruit crop which is well adapted to the tropical and subtropical agro-climatic conditions. Ochratoxin A (OTA) is an effective carcinogenic mycotoxin produced by *Aspergillus* fungi contaminating on grapes and grapes by-products like fresh grapes, raisin and wine. In the present study, survey was conducted in local markets of various districts in Tamil Nadu to identify the presence of OTA producing fungi on fresh grapes, black raisin and raisin. The mycoflora assessment identified that 90 per cent of all the three samples were contaminated with *Aspergillus niger, Aspergillus flavus, Rhizopus spp.* and *Penicillium spp*. whereas the maximum *A. niger* colonies were produced in black raisin (25.66 ± 0.57) sample NgBRS2 collected from Nilgris district and grape (5.53 ± 0.44) sample TGM2 collected from Theni district. The morphological studies on confirmation of the maximum colony producing *A. niger* isolates (MRL1, NgBRS2, TGM2) revealed that the growth pattern of the colony, colour of mycelia was similar and the microscopic observations showed that the three isolates of *A. niger* have septate hyphae bearing dark conidiophore with globose vesicle producing acropetally dark brown coloured chain of conidia and whereas the metulae, phialides and vesicle were covered with mass of conidia. The maximum colony producing virulent strain NgBRS2 was subjected to molecular identification using the ITS 1 and ITS 4 transcribed region amplified 18S-28S rRNA gene fragment of *A. niger* at ~570 bp and the accession number for nucleotide sequence was obtained (OK284581). The HPLC analysis on OTA detection showed the potentiality of NgBRS2 *A. niger* strain in OTA production. The study provides the importance of potential carcinogenic fungal and OTA contaminations on grapes and its by-products in markets of Tamil Nadu which may oblige the future research on ochratoxigenic fungi and OTA detoxification.

Keywords: Grapes, raisins, ochratoxin, *Aspergillus niger,* Mycotoxin, HPLC

1. Introduction
Grapes and raisins are energy fruits that are grown in almost every part of the world. Grapes and raisins are rich in fibre, potassium and a variety of minerals and vitamins. The grape itself used as myriad of products like pre-serves, squashes, wines & related fermented products and raisins. Total area under grapes in India is about 1,38,900 ha with the production of 29,20,100 MT, distributed mainly in Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu (Horticulture Statistics, 2018). The area dedicated to vineyards is increasing by about 2 per cent per year. The nutrients in grapes offer a number of health benefits but the grapes and grape derived products are attacked by various fungal organism that affect the quality of products.

Ochratoxin A is an important mycotoxin commonly found in cereals, grapes, cocoa, spices, coffee and their processed product. Ochratoxin A (OTA) is a potential mycotoxin synthesized mostly by *Aspergillus* and *Penicillium* species. *A. niger* and *A. carbonarius* are the most common potent Aspergillus species contaminating grapes during veraison to harvest. In addition to deterioration of fruits, this fungus also leads to the production of ochratoxin the common mycotoxin present in grapes and grape derivatives such as raisins, wine, squashes and juices.

Grapes and grape-derived products are a key source of OTA in the diet for persons who consume a lot of grapes (Akdeniz *et al.*, 2013) [3]. This mycotoxic fungi has nephrotoxic, genotoxic, teratogenic and carcinogenic properties (Rocha *et al.*, 2014) [25]. Ochratoxin A has been designated as a potential human carcinogen by the International Cancer Research Agency under category 2B (IARC, 1993) [13]. The European Union set a limit of 5 to 10 µg/kg for OTA content in cereals, coffee, grapes and grape-derived products and 2 µg/kg for grape juice and wine in various food products. (Wei *et al.*, 2017, Akdeniz *et al.*, 2013) [3].

Based on the circumstances, the present study aimed to assess the mycoflora and detection of Ochratoxin A in grapes and raisins collected from different districts of Tamil Nadu.

2. Materials and Methods

2.1. Grapes Sampling

A total of sixty samples of grape and grape derived products raisin (n=25), black raisin (n= 20) and grapes (n=15) were collected randomly from local dealers and grape fields at different location in Tamil Nadu, India during 2020-21. The samples were collected in sterilized polythene bags, labelled and stored at 4 °C for further studies.

2.2 Mycoflora assessment

The microbial population of grape samples (Raisins, Black raisins and Grapes) were enumerated as described by Abdel-Sater and Eraky (2001) [2] with slight modification. One gram of each sample was crushed and serially diluted with sterile distilled water. One ml aliquot was plated on Rose Bengal chloramphenicol agar media (Peptone 5g/L, Dextrose 10g/L, Potassium dihydrogen phosphate 1g/L, Magnesium sulphate 0.5g/L, Rose Bengal 0.05g/L, Chloramphenicol 0.1g/L, Agar 20g/L, pH 7.2). After that, plates were incubated at 28°C for 72 to 96hrs. Fungal population was expressed as CFU/g. Pure culture was obtained by subsequent culturing on YES medium (Yeast extract 4g/l, Sucrose 20g/l, KH2PO4 1.0g/l, MgSO4 0.5g/l, Agar 20g/l) and maintained at 4 °C for further studies.

2.3 Identification of black ochratoxigenic fungi

The fungal isolate was characterized morphologically with the help Phase contrast microscope. Based on the mycelial, spore and conidial heads characters observations, the fungal colonies were identified morphologically (Fig. 1).

Morphologically identified *Aspergillus niger* was subjected to molecular characterization by ITS. The fungal isolates were grown in YES broth for five days and DNA from each fresh mycelial mat were extracted by CTAB method. The DNA of each samples were subjected to PCR amplification of the ITS region ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. The PCR products were sequenced and the respective nucleotide sequences were blasted in NCBI and compared with the reference sequences of other *Aspergillus* isolates from GenBank database. The sequence of each fungal isolate was submitted individually in NCBI database for accession number.

2.4 Detection and quantification of Ochratoxin A (OTA) produced by *Aspergillus niger* by HPLC analysis

The ochratoxin A (OTA) production of the virulent *A. niger* strain (NgBRS2) was analysed according to protocol reported by Bragulat *et al*. (2001) with slight modifications. The NgBRS2 strain was cultured on YES medium and three agar plugs of *A. niger* were removed aseptically from the internal, middle and external areas of the well grown fungal colony at 10th day after incubation (DAI) under 25 °C temperature. For OTA extraction, the agar plugs were transferred to sterile Eppendorf 2 ml vial and one ml of HPLC grade methanol was added. The mixture was homogenized by vortexing for one min and followed by 60 mins incubation. The mixture was then centrifuged at 10,000 rpm for 5 mins. The supernatant was separated carefully and filtered using Millex® syringe filter (0.22 µm). The methanol extract was dried under nitrogen gas at 35 °C and re-dissolved in mobile phase (Acetonitrile: Water: Acetic acid 51:47:2). The sample was injected into a High-performance liquid Chromatography (HPLC) instrument (Agilent 1200 HPLC system, Agilent Technologies, USA).

Ochratoxin were determined by reverse- phase HPLC (Agilent 1200 HPLC system, Agilent Technologies, USA) equipped with fluorescence detector and auto sampler. The silica packed C18 column (150mm × 4.6mm, 5µm particle size; Agilent Technologies, USA) was used for chromatographic separation was maintained at 40 °C. The mobile phase was prepared with the mixture of acetonitrile, water and acetic acid (51:47:2). The flow rate of mobile phase was maintained at 1 ml/ min. The excitation and emission wavelength of FLD detection were 333 and 443 nm respectively. Fifty microlitre from each sample was used as an injection volume. The tested fungi were taken as positive OTA producer if the peak of sample was obtained at a retention time similar to the peak of OTA standard, with a height five times higher than the baseline noise. Limit of detection (LOD) was of 0.7 ng ml^{-1}.

2.5 Statistical analysis

Data of infection per cent was subjected to analysis of variance (ANOVA) and mean were compared using Least Significance Difference (LSD) at 5% probability level (Gomez & Gomez, 1984).

3. Result

3.1 Mycoflora assessment in raisin, black raisin and grapes

The mycoflora assessment of raisin, black raisin and grape samples collected from various districts of Tamil Nadu showed significant differences among the mycoflora population present on each respective product. The study on mycoflora assessment revealed that, more than 90 per cent of the samples collected were contaminated with *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus spp.* and *Penicillium spp* (Table. 1, 2 & 3). Similarly, the study on microbial isolation percentage showed 92 per cent of raisin, 90 per cent of black raisin and 73.33 per cent grape samples were contaminated by *A. niger.*

Further studies of mycoflora population assessment (cfu/g) on the raisin samples showed that the raisin sample MRL1 collected from Madurai local market produced maximum fungal colonies (cfu/g) of black fungi *A. niger* (6.6 ± 0.23) and other species of *A. flavus* (2.42±0.03), *Rhizopus spp* (1±0) and *Penicillium spp* (0.67±0) respectively. The maximum *A. niger* colonies were observed in black raisin (25.66 ± 0.57) sample NgBRS2 collected from Nilgris district and grape (5.53 ± 0.44) sample TGM2 collected from Theni district (Table. 4, 5, & 6).

3.2 Morphological and molecular identification of black ochratoxigenic fungi

The colony characters of all the three isolates of *Aspergillus niger* (MRL1, NgBRS2, TGM2) with maximum CFU/g of Raisin, Black raisin and Grapes were studied on YES medium (Fig. 1)**.** All the three isolates collected from different regions of Madurai, Nilgris and Theni showed similar colony character *viz*., growth pattern of the colony, colour of upper and lower side of the culture plate were recorded on YES medium after 7 days of incubation at 25 °C. With respect to colour of the mycelia the isolates showed similar mycelial colour of dark black to brown spores on white mycelia, the

colony initially occur white in colour, but become black in few days due to rapid production of conidial spore according to macroscopic observation. And at the same time there was slight yellowish tinge on white mycelia initially which was occur due to radial cracks in mycelia.

The microscopic observations of all the three isolates of *A. niger* revealed that it produces coloured conidia and conidiophores on hyaline, septate hyphae. The conidiophore occurs dark at the apex and terminates with globose vesicle. Conidia are dark brown coloured, the metulae, phialides and vesicle are covered with conidial spores. The observation on pigmentation of *Aspergillus* revealed that the pathogen produced brown coloured pigmentation on posterior of the petri plate. All the three strains show similar brown coloured with wrinkled mycelia and rough texture (Fig 2).

PCR amplification of ITS region in the virulent isolate (NgBRS2) were performed using the universal primers of forward ITS 1 and ITS 4 amplified 18S-28S rRNA gene fragment of *A. niger* at 570 bp (approx) for all the isoaltes when visualized under UV light (Fig. 3). The gene fragments of all the four isolates were sequenced and submitted in NCBI database. The nucleotide sequence of 18S-28S rRNA gene was 99.80 percent similar to that of *A. niger* strain submitted in NCBI (OK284581).

3.3 Detection of Ochratoxin A by HPLC

The OTA production of *Aspergillus niger* strain NgBRS2 was significantly detected through HPLC analysis. The results revealed that the toxin extracted from *A. niger* exhibited the peak at 4.824 mins retention time which was similar to peak produced by the OTA standard at 4.838 mins RT (Fig. 4).

4. Discussion

4.1 Mycoflora assessment on raisin, black raisin and grape samples

The tropical climatic condition of Tamil Nadu, India favours the development *Aspergillus sp. Penicillium and Rhizopus.* Of the total sixty samples (raisins (n=25), black raisins (n=20) and grapes (n=15)) collected from different districts of Tamil Nadu were found to be dominated by *Aspergillus niger* than any other species which affect grapes mainly during ripening to harvest stage (Table. 1). Comparatively, the overall study on mycoflora contamination on grapes and grapes products found that the black raisin samples were highly contaminated by ochratoxigenic fungi *A. niger* which is a rigours OTA (Ochratoxin) producer. The production of maximum colonies of *A. niger* on Nilgris black raisin sample may be attributed to the spore producing ability of the NgBRS2 *A. niger* strain. The maximum spore production may be directly proportional to the toxin production also.

Similarly, Chebil *et al*. (2020) [8] identified the occurrence mycobiota from dried grapes samples and found the presence of *Aspergillus niger* aggregate (73%), *Aspergillus carbonarius* (10%), *Aspergillus japonicus* (10%), *Aspergillus flavus* (8%) and *Aspergillus ochraceus* (1%). Whereas the *Aspergillus niger* aggregate were the most predominantly found isolates ranging 70, 80 and 85 per cent in dried grapes samples from the markets of Kelibia, Sfax and Rafraf, respectively and 100 per cent of the relevant mycobiota observed in imported dried grapes samples. Also, identified the presence of the genera *Penicillium*, *Alternaria*, *Botrytis* and *Rhizopus* in dried grapes. Among the potential OTA producing fungi, only black *Aspergilli* represented by *Aspergillus* section Nigri group and *Aspergillus ochraceus* were isolated from the analyzed samples, while no isolates of *Penicillium verrucosum* were identified.

The *A. niger* aggregate was predominant within the *Aspergillus* section *Nigri*, representing 78 per cent of the isolates (Battilani *et al*., 2003; Khoury *et al*., 2006 and Ponsone *et al*., 2010) [4, 14, 24]. The *Aspergillus niger* species are considered as the main *Aspergillus* contaminant in grapes produced even in regions with climate conditions that are completely different, probably because these contaminating fungi are great competitors having good CSA and can be extremely adapted to the ecosystem present in the vineyards. The aggregate of *A. niger* species represents 80 to 85 per cent of the contamination, mainly during harvest period (Visconti *et al*., 2008) [30].

In warmer climate countries, like India, the microorganism like *Botrytis*, *Alternaria* and *Rhizopus spp*. are the most abundant fungi at the beginning of grapes ripening process. The genus *Aspergillus* and *Penicillium* are the most common post-harvest fungi contaminating grapes during the late stages of ripening, harvest and during solar drying of grapes, which are capable of mycotoxin production like OTA (Abarca *et al*., 2003; Belli *et al*., 2005; García-Cela *et al*., 2015; Oliveri and Catara, 2011; Valero *et al*., 2005) [1, 6, 11, 18, 29]. During this post-harvest treatment, grapes are dried until their sugar level is extremely high, but at the same time, this process can produce adverse effects in the fruit mycoflora like an increase of fungal development and OTA production (Bau *et al*., 2005; Merlela *et al*., 2015; Palumbo *et al*., 2015; Pardo *et al*., 2005) [5, 17, 20, 21].

4.2 Identification of black ochratoxigenic fungi

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. The micrometry observation of *A. niger* strain obtained from raisin, black raisin and grape showed black coloured condia present on conidiophore embedded hyaline, septate hyphae. Similarly, Klich *et al*. (2002) [15] reported that the strains belonging to the genus Aspergillus Section Nigri characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae near the apex.

Silva *et al*. (2004) reported that the spore ornamentation as observed in MEV permitted the distinction of two groups of the analyzed species of Aspergillus Section *Nigri*: those that presented warty conidia and those that presented echinulated conidia. The species that present warty conidia were: *A. niger*, *A. niger* aggregate, *A. carbonarius* and *A. tubingensis*.

The mycelial growth characterisation of the *A. niger* strain produces white coloured mycelia initially but later turn black due to rapid production of spores. Likewise, Silva *et al.* (2011) [28] presented the growth characteristics of the species *Aspergillus* Section *Nigri* strain UFLA DCA 01 cultured on CYA (Czapeck Yeast Agar) and MEA (Malt Extract Agar) at 25 ºC incubated for seven days and reported that the *Aspergillus* strain found have low sporulation capacity and abundant production of oval shaped yellowish orange sclerotia with grey tones. Accordingly, Bernice *et al.* (2015) [7] reported that the *A. niger* produced yellowish black colonies on CYA media and *A. tubingensis* strain was morphologically very similar to *A. niger*. The morphological identification showed the significance in physical and morphological variability among the Ochratoxigenic fungal aggregate isolated from Grapes products.

4.2.3 Molecular Characterization of *A. niger*

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) [27]. The present study on PCR analysis of the *A. niger* strain (NgBRS2) with ITS universal primer were amplified at 570bp. Further the nucleotide was sequenced and compared with *A. niger* strains in NCBI database. The DNA sequence of *A. niger* strain NgBRS2 showed similarity of 99.80 per cent was submitted and obtained accession number (OK284581).

Similarly, Gautam and Bhadauria (2012) [12] carried out molecular characterization and identified the frequently isolated six species of *Aspergillus* with the help of fungal universal primer ITS-4.

Correspondingly, Oliveri (2016) [19] used the primer combination ITS1/NIG and identified *A. niger* aggregate strains by amplifying 420 bp genomic DNA from the 88 previously morphologically identified fungus and CBS 127.49) were conspecific. Similarly Esteban *et al.* (2006) sequenced ITS1-5.8S–ITS2 rDNA region and Obtained the phylogenetic tree of five *A. carbonarius* strains (M325, CBS 110.49, NRRL 67, A-941 and CBS 127.49) were conspecific.

Sequence analysis of the ITS regions was proved to be a suitable tool to separate *A. carbonarius* isolates from other black *Aspergilli* (Parenicova *et al*., 2000) [22].

4.4 Detection of OTA production in *A. niger*

The HPLC analysis confirmed the presence of OTA production of virulent *A. niger* strain NgBRS2 isolated from Black raisin samples collected from the Nilgris district of Tamil Nadu. The OTA production of virulent *A. niger* strain may be attributed with that of vigorous colony multiplication at environmental factors in hilly region compiled with more water activity. Several researchers have confirmed the vital role of stage of growth, temperature, humidity and water activity in OTA production.

Cabanes *et al*. (2002) [9] analyzed the OTA production by two *A. niger* isolates and five *A. carbonarius* isolates grown at 25 ºC on yeast extract sucrose agar (YES). Maximum OTA production was seem to be depend on the isolates tested and not by the group aggregate. Belli *et al.* (2004) reported that maximum quantity of ochratoxin were found at the earlier growth states of *A. niger* isolates tested is an important hazard for OTA contamination in grapes. Similar reports showed the significant amount of OTA produced after 5th day of incubation, but the maximum OTA production observed at 25 ºC between 7th to 14th day of incubation (Esteban *et al*., 2004; Bellı´ *et al*., 2004). Patharanjan *et al.* (2011) reported that different ecological parameters have greater impact on OTA production by *Aspergillus* fungus which can be useful obtaining knowledge on OTA contamination and to develop proper management strategies in future research programmes.

Table 1: Microbial isolation frequency of raisin samples collected from various markets of Tamil Nadu

S. No	Sample code	*A. niger*	*A. flavus*	*Rhizopus*	*Penicillium*
1	CRL1	+	+	+	-
2	CRL2	+	+	-	-
3	CRS3	+	+	-	-
4	SRL1	+	+	-	+
5	SRL2	+	+	+	-
6	SRS3	-	-	-	-
7	ERL1	+	+	-	-
8	ERS2	-	+	+	-
9	NRL1	+	+	-	-
10	NRS2	+	+	-	-
11	DRL1	+	+	-	-
12	MRL1	+	+	+	+
13	MRS2	+	+	-	+
14	TkRL1	+	+	+	-
15	TkRS2	+	+	-	-
16	CnRL1	+	+	-	+
17	NgRL1	+	+	-	+
18	NgRS2	+	+	-	-
19	TjRL1	+	+	+	-
20	TiRL1	+	-	-	+
21	TiRS2	+	-	-	+
22	TpRL1	+	+	-	+
23	KRL1	+	+	-	+
24	KRS2	+	+	+	+
25	KkRL1	+	+	-	+

Table 2: Microbial isolation frequency of black raisin samples collected from various local markets of Tamil Nadu

S. No.	Sample code	*A. niger*	*A. flavus*	*Rhizopus*	*Penicillium*
1	CBRL1	+	+	-	-
2	CBRS2	+	+	-	-
3	CBRS3	-	+	-	+
4	SBRL1	+	-	-	-
5	SBRL2	+	-	-	-
6	SBRS3	+	+	-	-

7	MBRS1	+	+	-	-
8	CnBRL1	+	+	-	-
9	CnBRS2	+	+	-	-
10	KBRL1	+	+	-	-
11	KBRL2	+	+	-	+
12	KBRS3	+	+	+	+
13	TpBRL1	+	+	+	-
14	TpBRS2	+	+	-	-
15	NgBRS2	+	+	-	-
16	NgBRS3	+	+	-	-
17	TkBRL1	+	+	-	-
18	TkBRS2	+	+	+	-
19	KkBRL1	-	-	-	-
20	KkBRS2	+	+	-	-

Table 3: Microbial isolation frequency of grapes samples collected from various markets of Tamil Nadu

S.no.	Sample code	*A. niger*	*A. flavus*	*Rhizopus*	*Penicillium*
1	CGF1	+	+	+	+
2	CGF2	+	+	+	+
3	CGF3	-	-	-	-
4	CGF4	-	-	-	-
5	CGF5	-	-	+	+
6	CGM6	+	+	-	+
7	CGM7	+	+	-	-
8	CGM8	+	-	+	+
9	CGM9	+	+	+	+
10	TGF1	+	+	+	+
11	TGF2	-	-	-	+
12	TGF3	+	+	-	-
13	TGM1	+	+	-	+
14	TGM2	+	+	-	+
15	TGM3	+	-	-	-

Table 4: Enumeration of total fungi from Raisin samples of Tamil Nadu

Average mould count *10^4 (CFU/g of raisin)					
S. No.	**Sample code**	***A. niger***	***A. flavus***	***Rhizopus***	***Penicillium***
1	CRL1	5.83±0.47 (3.76)	1.16±0.1 (3.06)	0.66±0.14 (2.81)	0
2	CRL2	0.36±0 (2.12)	0.12±0 (2.07)	0	0
3	CRS3	0.53±0 (1.52)	0.17±0.08 (2.23)	0	0
4	SRL1	0.3±0.18 (2.63)	0.19±0.03 (2.27)	0	0.26±0.02 (2.41)
5	SRL2	0.76±0.35 (2.95)	1.36±0.28 (3.13)	3.33±0.60 (3.52)	0
6	SRS3	0	0	0	0
7	ERL1	3.4±0.86 (2)	0	0	1.23±0 (3.08)
8	ERS2	0	1.22±0.1 (3.08)	0.66±0.33 (2.81)	0
9	NRL1	4.4±0 (3.64)	0.13±0 (2.11)	0	0
10	NRS2	0.5±0 (2.69)	0.16±0 (2.20)	0	0
11	DRL1	0.4±0 (2.60)	0.13±0 (2.11)	0	0
12	MRL1	6.6±0.23 (3.81)	2.42±0.03 (3.38)	1±0	0.67±0 (2.82)
13	MRS2	5.4±0.61 (3.7)	0.13±0 (2.11)	0	3.7±0 (3.56)
14	TkRL1	0.43±0.47 (2.63)	0.36±0.28 (2.55)	0.66±0.28 (2.81)	0
15	TkRS2	0.46±0 (2.66)	1±0.03 (3)	0	0
16	CnRL1	1.4±0.12 (3.1)	1.3±0.03 (3.11)	0	3±0 (3.47)
17	NgRL1	0.4±1.76 (2.60)	1.3±0 (3.11)	0	2.5±0 (3.39)
18	NgRS2	0.86±0 (2.93)	2.8±5.6 (3.44)	0	0
19	TjRL1	0.46±4.71 (2.66)	2.37±0.03 (3.37)	6.66±0.33 (3.82)	0
20	TiRL1	0.6±0.07 (2.77)	0	0	1±0 (3)
21	TiRS2	0.03±0 (1.47)	1±0.07 (3)	0	0
22	TpRL1	2.13±0.24 (3.32)	0.82±0 (2.90)	0	0.34±0.32 (2.53)
23	KRL1	0.83±0.00 (2.91)	2.78±0 (3.44)	0	3±0.003 (3.47)
24	KRS2	0.83±0.94 (2.91)	2.83±0.33 (3.45)	7.33±0.29 (3.86)	3.4±0.33 (3.53)
25	KkRL1	4.23±1.13 (3.62)	1.8±0.48 (3.25)	0	1.6±0.17 (3.20)

Values in the column represent means of triplicate determinations ± SE (Standard Error),
cfu – Colony Forming Unit
Values in parenthesis are log ^{10} transformed value

Table 5: Enumeration of total fungi from Black Raisin samples of Tamil Nadu

Average mould count *10^4 (CFU/g of Black Raisin)					
S. No	**Sample code**	***A. niger***	***A. flavus***	***Rhizopus***	***Penicillium***
1.	CBRL1	19.3±0.8 (4.28)	16.93±0.35 (4.22)	0	0
2.	CBRS2	27.26±0.63 (4.43)	17.63±0.63 (4.24)	0	0
3.	CBRS3	0	0.73±0 (2.86)	0.13±0.13 (2.11)	0
4.	SBRL1	2.97±0 (3.47)	0	0	0
5.	SBRL2	1.05±0 (3.02)	0	0	0
6.	SBRS3	3.53±1.28 (3.54)	1.43±0.29 (3.15)	0	0
7.	MBRS1	6.3±2.90 (3.79)	9.2±0.35 (3.96)	0	0
8.	CnBRL1	3.8±1.44 (3.57)	13.8±0.178 (4.13)	0	0
9.	CnBRS2	14.33±1.73 (4.15)	4.53±0.33 (3.65)	0	0
10.	KBRL1	15.5±1.10 (4.19)	13.2±0.28 (4.12)	0	0
11.	KBRL2	2±0.29 (3.30)	1.46±1 (3.16)	0	1.66±0.67 (3.22)
12.	KBRS3	2.36±0.46 (3.37)	0.46±0.52 (2.66)	0.96±0.97 (2.98)	0.46±0.2 (2.66)
13.	TpBRL1	13.26±0.46 (4.12)	0.46±0.63 (2.66)	0.8±0.8 (2.90)	0
14.	TpBRS2	8.06±2.44 (3.90)	8.06±0.50 (3.90)	0	0
15.	NgBRS2	25.66±0.33 (4.40)	2.66±0.60 (3.42)	0	0
16.	NgBRS3	17.2±1.0 (4.23)	0.5±0.3 (2.69)	0	0
17.	TkBRL1	19.6±0.78 (4.29)	3.9±0.57 (3.59)	0	0
18.	TkBRS2	27.4±0.50 (4.43)	7.2±0 (3.85)	0.5±0.35 (2.69)	0
19.	KkBRL1	0	0	0	0.2±0 (2.30)
20.	KkBRS2	5.2±0.3 (3.71)	1.3±0.3 (3.11)	0	0

Values in the column represent means of triplicate determinations ± SE (Standard Error),
cfu – Colony Forming Unit
Values in parenthesis are log ^{10} transformed value

Table 6: Enumeration of total fungi from Grape samples of Tamil Nadu

Average mould count *10^4 (cfu/g of Grapes)					
S. No.	**Sample code**	***A. niger***	***A. flavus***	***Rhizopus***	***Penicillium***
1	CGF1	3.6±0.14 (3.55)	2.76±0.70 (3.44)	0.33±0.01 (2.51)	0.43±0.03 (2.63)
2	CGF2	0.1±0.05 (2)	0.1±0.05 (2)	0.43±0.29 (2.63)	3±0.57 (3.47)
3	CGF3	0	0	0	0
4	CGF4	0	0	0	0
5	CGF5	0	0	3±0.57 (3.47)	2.26±0.14 (3.35)
6	CGM6	0.73±0.17 (2.86)	0.93±0.26 (2.96)	0	0.2±0.1 (2.30)
7	CGM7	1.23±0.08 (3.08)	1.23±0.12 (3.08)	0	0
8	CGM8	2.13±0.14 (3.32)	7.76±0.16 (3.88)	0.83±0.16 (2.91)	1±0 (3)
9	CGM9	3.5±0.20 (3.54)	2.6±0.28 (3.41)	0	0.83±0.08 (2.91)
10	TGF1	3.9±0.1 (3.59)	5.4±0.05 (3.73)	2±0 (3.30)	0.76±0.08 (2.88)
11	TGF2	0	0.03±0 (1.47)	0	7±0.57 (3.84)
12	TGF3	3.33±0.05 (3.52)	0	2.96±1.18 (3.47)	6.16±0.44 (3.17)
13	TGM1	2.33±0.66 (3.36)	0.6±1.85 (2.77)	0.66 (2.81)	0.33±0.33 (2.51)
14	TGM2	5.53±0.08 (3.74)	3.03±1.98 (3.48)	2±1.98 (3.30)	3.13±0.13 (3.49)
15	TGM3	0.1±0.03 (2)	0.06±0.1 (1.17)	0.06±0.06 (1.77)	0.03±0 (1.47)

Values in the column represent means of triplicate determinations ± SE (Standard Error),
cfu – Colony Forming Unit
Values in parenthesis are log ^{10} transformed value

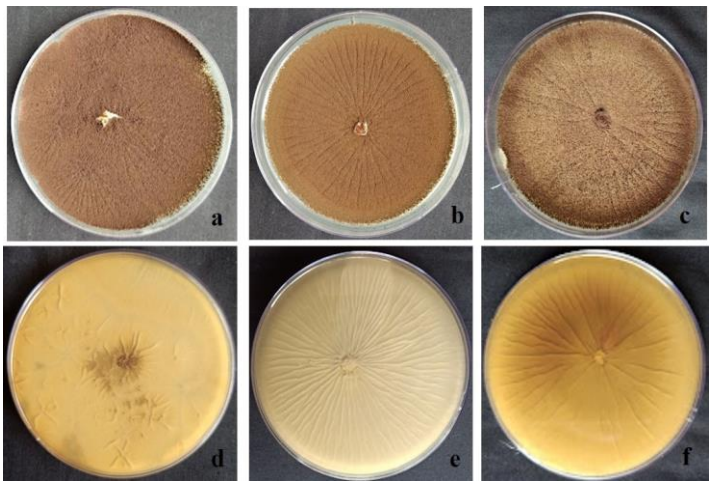

Fig 1: Morphological difference between *A. niger* isolates from a raisins, black raisin and grapes samples collected from different districts of Tamil Nadu a) MRL1, b) NgBRS2 and c) TGM2; d, e, f) lower surface of the respective *A. niger* colony isolates

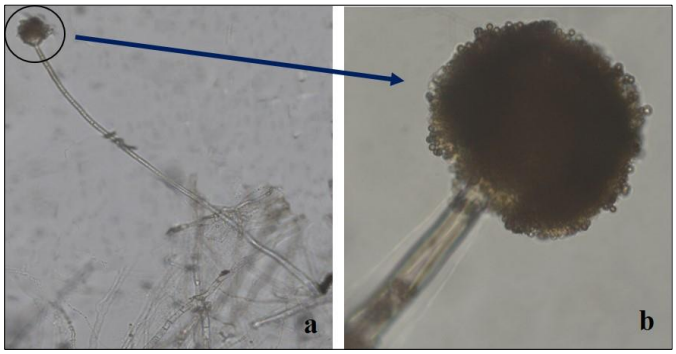

Fig 2: Microscopic observation of *A. niger* a) Mycelia and conidiophore structure b) Conidial orientation

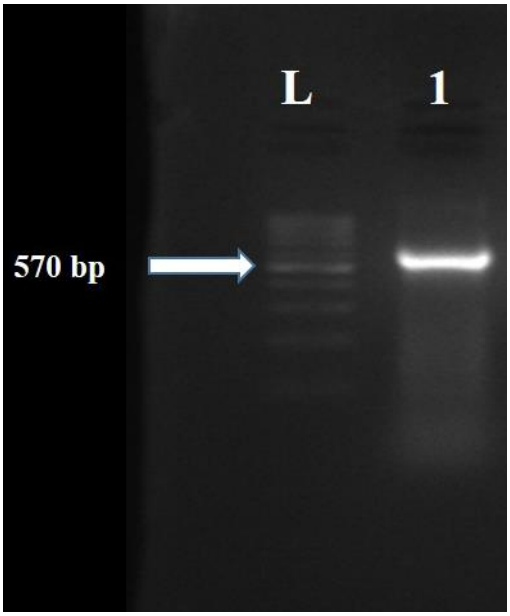

Fig 3: PCR amplification of *A. niger* (NgBRS2) 18S DNA region using ITS- 1 and ITS- 4 primers

Where
L: 100 bp ladder
1: DNA replicates of NgBRS2 strain

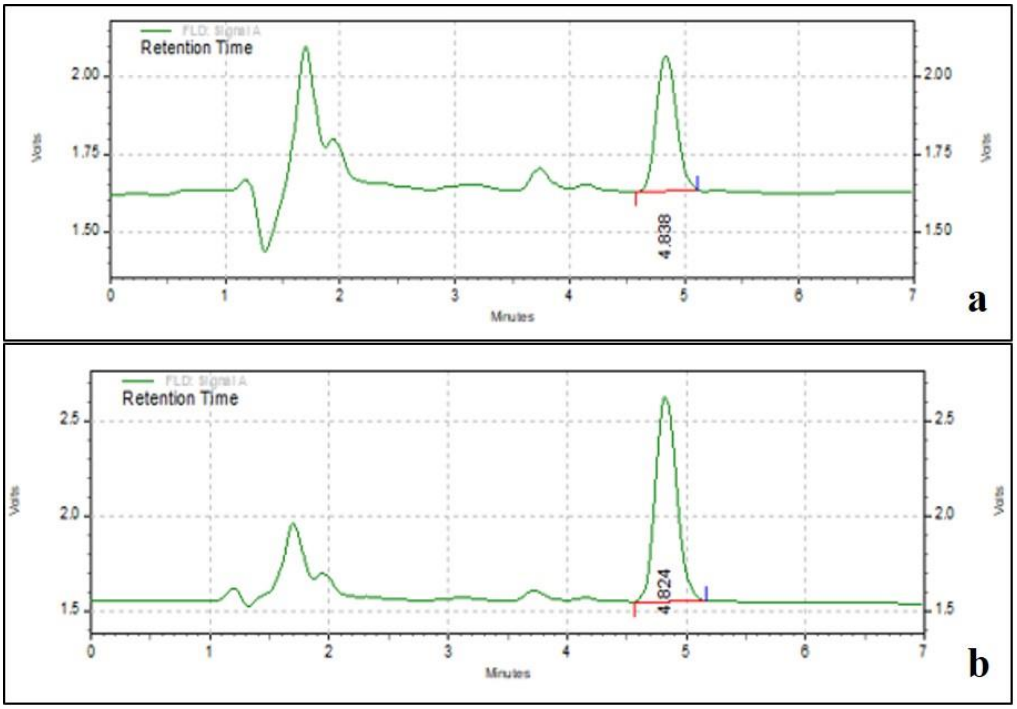

Fig 4: HPLC chromatograms showing OTA detection a) standard 10 µg/g B) *A. niger* isolate (NgBRS2) after 10 days of incubation

5. Conclusion

To our knowledge only limited attempts have been made for studying the Ochratoxin A (OTA) and Ochratoxigenic fungi on grapes and grapes-by products in Indian sub-continent. Besides there is necessity for a common basic knowledge on OTA producing fungi and their ability to produce carcinogenous toxins as a preliminary data to overcome the negative impact on human health. According to our results, the grapes and grapes by-products *viz.*, raisins and black raisins were contaminated by toxigenic fungi, especially ochratoxigenic fungi like *A. niger, A. flavus and Penicillium spp*. The *A. niger* was found to be ubiquitous on the Black raisin samples in the Nilgris local market and similarly the strain NgBRS2 was a potential OTA producer under crucial eco-physiological condition. Minding on the ecophysiological factors of the particular place sensible choosing of the harvesting dates and minimized harvest and transport time to post harvest processing units become crucial. Moreover, any physical damages on the grape skin may prompt the entrance of the contaminating fungi that colonized the surface that may probably enable the maximum quantity of OTA production in grapes.

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