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Enumeration of Fungal Contaminants in Rice and characterization of Ochratoxigenic Fungi *Aspergillus* spp

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ABSTRACT: Rice (*Oryza sativa* L.), India's most prominent cereal crop and a staple food for Indian population. Rice is susceptible to a broad spectrum of fungi, the infection of which is associated with quality deterioration and toxin production. Totally ninety samples encompassing (nine paddy and eighty-one brown and polished rice) were collected from markets of various rice growing districts of Tamil Nadu to assess fungal contaminants and occurrence of ochratoxigenic fungi particularly *Aspergillus* spp. The study documented *Aspergillus*, *Penicillium*, and *Fusarium* were the dominant fungal genera with highest population of 8.24×10^3–6.03×10^6 CFU/g was recorded by *Aspergillus* sp in paddy samples. The isolates of *Aspergillus niger* and *Aspergillus ochraceus* was identified based on colony morphology on Czapek Yeast Extract (CYA) medium incubated for 15 days under dark and by microscopic observation. Further, isolates of *A.ochraceus* were confirmed molecularly by the amplification of polyketide synthase gene (pks) using species specific primer AoLc35F, AoLc35R. The PCR amplification resulted with an amplicon size of 520 bp which confirmed the isolates are *Aspergillus ochraceus* and found to be ochratoxin A producer.

Keywords: Rice, *Aspergillus niger*, *A. ochraceus*, Czapek Yeast Extract (CYA) medium, ochratoxin A (OTA).

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal crop and millions of small farmers; landless labourers depend on rice cultivation for their income generation. Among total rice production, human consumption accounts for 85% compared to wheat and maize (Mazaheri, 2023). The major rice producing states in India are West Bengal, Uttar Pradesh, Andhra Pradesh, Punjab, Tamil Nadu, Bihar, and Odisha which contribute 72% of total rice output. In Tamil Nadu, cultivable area was around 2.04 million hectares, with production of 7.98 million metric tons contributing 6% of India's total rice production. Globally, India was the largest rice exporter of 16.5 million metric tonnes and Bangladesh, United Arab Emirates were the major importers of Indian rice. In India, about 10% of the agricultural GDP comes from rice sector (Singh *et al*., 2021).

Rice which is cultivated commonly under flood irrigation and prevailing high moisture level promote the fungal growth. The fungal contamination may occur at pre harvest stage in the field or during post-harvest such as transport, inadequate drying and improper storage condition (Reddy *et al*., 2008). The predominant mycotoxigenic fungi in rice belong to the genera are *Aspergillus* sp., *Fusarium* sp and *Penicillium* sp. In rice samples, 80% of the fungal isolates belonged to the genera *Aspergillus* and 20% were *Penicillium* (Laut *et al*., 2023). As per the report of Trung *et al*. (2001) 43.8% *Aspergillus* sp., 21.9% *Fusarium* sp., 10.9% *Penicllium* sp., and 23.4% of other fungi were found in rice samples.

Ochratoxin A producing fungi that can contaminate rice primarily belongs to the genera *Aspergillus* and *Penicillium* (Lee and Ryu 2015). The major producers of OTA belonging to the genera *Aspergillus*, *Penicillium* including species such as *A. ochraceus, A. carbonarius, A. niger*, and *P. verrucosum* (Ben Miri *et al*., 2024). Several authors reported the investigation of isolates of *A. niger* in rice samples at different countries. Accordingly, Trung *et al*. (2001) in Vietnam; Shanakht *et al*. (2014) in Lahore; Ranjbar *et al*. (2019) in Khuzestan. The ochratoxigenic fungi which was isolated from Korean polished rice was *A. niger*, *A. ochraceus* and *P. verrucosum* (Park *et al*., 2005). Hafez *et al*. (2022) identified the prevalence of *A. ochraceus* in rice samples of Egypt.

The fungi *A. niger* and its closely related species (*A. carbonarius*, *A. awamori*, *A. aculeatus* and *A. japonicus*) all belong to *Aspergillus* Section *Nigri* and the colonies of *A. niger* are black or dark brown in colour (Perrone *et al*., 2007). The predominant OTA producer in *Aspergillus* Section *Circumdati*is *A. ochraceus* produces yellow brown (ochre) colonies (Visagie *et al*., 2014). Further molecular techniques are developed to distinguish *Aspergillus* spp (Esteban *et al.,* 2006). The species-specific PCR-based assays to differentiate ochratoxigenic species were developed based on ITS region for *A. ochraceus* (Patino *et al*.,

2005). In *A. ochraceus*, the sequence of AoLC35-12 encode for acyl transferase domain of a polyketide synthase, which is involved in the OTA biosynthesis pathway (Atoui *et al*., 2006).

Under favourable condition, the ochratoxigenic fungi produce ochratoxin A (OTA) well-known fungal mycotoxins of worldwide concern. It was first isolated from *Aspergillus ochraceus* in south Africa by Van der Merwe *et al*. (1965), hence the name ochratoxin A (Li *et al*., 2021). Gonclaves *et al*. (2019) documented the prevalence of *A. ochraceus* in rice and its ability to produce OTA. OTA exhibits neurotoxic, hepatotoxic, immunotoxic, mutagenic, and teratogenic effects. OTA is also classified as possibly human carcinogen (group 2B) by IARC (International Agency for Research on Cancer) (Humans *et al*., 1993). The impact of *Aspergillus* infection and ochratoxin A production cause economic loss in rice production by reducing the market value of rice (Pandey *et al*., 2023). Further it decreases the nutritional quality of rice and cause harmful effects on animal and human health. Hence the present investigation was undertaken to assess the prevalence of fungal contaminants in rice and identification of ochratoxigenic fungi particularly *A. niger* and *A. ochraceus*.

MATERIALS AND METHODS

Chemicals used. For culture maintenance, sterile disposable plates (Himedia, Mumbai) were used and they were autoclaved twice at 121 °C for 15 minutes each before being discarded. Glasswares used for the experiments such as funnels, beakers, and Erlenmeyer flask was cleaned by immersing it in 1% NaOCl for two hours and then in 5% acetone for 30 minutes.

Collection of samples. The samples were collected from different rice growing districts of Tamil Nadu, India during 2022-23. A total of ninety samples including nine paddy and eighty-one brown rice, polished rice samples were obtained to assess the fungal contaminants and to isolate, identify the ochratoxigenic fungi particularly *Aspergillus* spp. The samples were brought randomly from petty shop, retail stores and whole sale markets of various districts *viz*., Coimbatore, Erode, Salem, Trichy, Thanjavur, Kanyakumari, Namakkal, Madurai, Dharmapuri, Dindigul, Karur, Tirupur, Theni, Kangayam, Nilgiris, Thoothukudito enumerate the fungal population and for identification of ochratoxin A producing fungi. The sampling size of 2 kg was obtained by pooling 10 cumulative samples of 200 g each. The samples were grounded to fine particle size using hand mill (BTC, India), labelled and stored at 4 °C for further experiments.

Assessment of fungal contaminants in paddy, brown rice and polished rice samples. The fungal population associated with paddy, brown rice and polished rice samples were enumerated using dilution plating technique as documented by Pitt and Hocking (1997). The finely grounded sample of one gram was taken in test tube containing ten ml of sterile distilled water. The test tubes were shaken for 30 min and were serially diluted with sterile water. Then, one ml of solution was plated on Rose-Bengal Chloramphenicol Agar medium and the plates were incubated at 30 ºC for three days to promote the growth of fungi. The fungal population on plates were expressed as colony forming units (CFU/g). Pure culture of fungal colony was obtained by single colony isolation method on PDA medium and maintained at 4 °C for further studies.

$$\text{CFU/g} = \frac{\text{Number of colonies} \times \text{Reciprocal of the dilution factor}}{\text{Volume of culture on plate}}$$

Identification of ochratoxigenic fungi *Aspergillus* spp

Morphological characterization. To identify the fungal genera, single hyphae were transferred to PDA amended Petri plates and incubated for 7 days at 30ºC. Subsequently, fungal colonies were transferred to Czapek Yeast Extract (CYA) (Sucrose 30 g, Yeast Extract 5 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, $ZnSO_4.7H_2O$ 0.1g, $CuSO_4.5H_2O$ 0.005 g Agar 15 g per 1 L)agar medium and incubated for 15 days at 25 ºC under dark. After the incubation period, colony morphology of fungi was observed and characteristics like colour (observe and reverse), shape, sporulation are used as a key description factor for identification (Pitt, 2009). The ochratoxigenic fungal isolates were characterized morphologically at 20x and 40x magnification with the help of Phase contrast microscope (Leica DM 2000 LED). The vegetative and sexual morphology was observed and imaged using software (LAS version 4.11.0) by Leica Microsystems (Switzerland) ltd.

Species confirmation of *A. ochraceus* using polyketide synthase gene (pks). The morphologically identified *Aspergillus* isolates were subjected to molecular characterization through the sequence analysis of 18S rDNA using ITS 1 and ITS 4 primers. Further *Aspergillus ochraceus* isolates were confirmed using AoLc35F, AoLc35R primer by the amplification of polyketide synthase gene (pks).

DNA extraction. DNA extraction was carried out from the fungal mycelial mat by adopting CTAB method prescribed by Priyadharshini *et al*. (2019). Five mm mycelial disc from 10days old *Aspergillus* colony was inoculated into 100 ml sterile potato dextrose broth and incubated at room temperature 28±2°C for seven days. After incubation, the mycelialm at was harvested and dried on filter paper. The mycelialm at was then ground finely with 3-5ml of CTAB buffer. The slurry (700µl) was then transferred to 1.5 ml centrifuge tube. The samples were incubated in water bath at 65 °C for 30 min and cooled at room temperature. To the extract 750 µl of phenol: chloroform: isoamyl alcohol (25:24:1) were added and centrifuged at 13,000 rpm for 15min.Thesupernatantwastransferredtonew1.5mlsterilecentrifugetube and 2/3 volume of ice-cold isopropanol was added and mixed gently. The samples were incubated at -20 °C overnight for the precipitation of DNA. After the incubation, the mixture was centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was discarded. The pellet was then washed thrice with 70 percent ethanol and centrifuged at 13,000 rpm for 5 m into remove impurities. The ethanol was discarded and the pellet was air dried for 30 min to remove traces of ethanol. Finally, the pellet containing DNA was

suspended in 50 µl of 1X TE buffer and stored at -20 °C. The purity and the concentration of the DNA was determined by spectrophotometrically at 260/230 nm using a nanodrop spectrophotometer (ND-1000, Wilmington, DE) and the total genomic DNA was checked by agarose gel electrophoresis.

Confirmation by PCR analysis using AoLc35F, AoLc35R primer. For species confirmation, *A. ochraceus* isolates were subjected to PCR amplification of pks gene using AoLc35F, AoLc35R primer. The PCR cycle followed for each reaction contains 35 cycles of initial denaturation at 94 °C for 4 min followed by denaturation at 94°C for 40S, primer annealing at 58°C for 40S, extension at 72 °C for 40 S.A final extension step was carried out at 72°C for 10min (Algammal *et al*., 2021). The amplified PCR products were sequenced by using sanger dideoxy sequencing method at Biokart, Bangalore. The identification of isolates was determined by comparing the acquired DNA sequences with the National Centre for Biotechnology and Information's (NCBI) database.

RESULTS AND DISCUSSION

Enumeration of fungal contaminants in paddy, brown rice and polished rice samples. The mycobiota study revealed different fungal genera belong to *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Rhizopus*, *Cladosporium*, *Mucor*, *Curvalaria*, *Trichoderma* and *Bipolaris* were documented in paddy, brown rice and polished rice samples. In line with our study, Pinciroli *et al*. (2013) documented the presence of *Alternaria, Bipolaris, Curvularia, Cladosporium, Penicillium* and *Fusarium* species in paddy, brown and polished rice samples. Egbuta *et al*. (2015), reported the rice samples were contaminated with fungus belonging to *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Rhizopus*, and *Cladosporium*. Similarly, Moharram *et al*. (2019) observed the prevalence of *Aspergillus, Penicillium, Alternaria, Cladosporium and Fusarium* in rice collected from the market at different localities of Egypt. The major fungi found in rice samples are *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Mucor*, *Rhizopus*, *Trichoderma*, *Curvularia*, *Helminthosporium* and *Cladosporia* (Makun *et al*., 2007). The occurrence of *Aspergillus* spp in rice was documented by Qi *et al.* (2022). Several authors reported the occurrence of common mycoflora associated with rice including *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Rhizopus* spp., *Alternaria* spp., *Mucor* spp (Phan *et al*., 2021); *Bipolaris* spp., *Fusarium* spp., *Aspergillus* spp., *Curvularia* spp., *Botryodiplodia* spp. (Ackaah *et al*., 2023); and *Alternaria alternata*, *F. moniliforme*, *Rhizopus nigricans* (Kumar *et al*., 2023).

In our current study, the highest fungal population in paddy (Table 1, Fig. 1a) was recorded by *Aspergillus* spp with a range of 8.24×10^3–6.03×10^6 CFU/g followed by *Penicillium* which showed 4.82×10^3–2.76×10^5 CFU/g and *Fusarium* with a range of 1.9×10^3–0.7×10^5 CFU/g. Further, the present study investigated that *Aspergillus*, *Penicillium* and *Fusarium* were the most dominant fungal genera recorded in paddy samples. In agreement with our study, Reddy *et al*. (2009) reported the paddy samples from India found to be infected with *Aspergillus* sp mainly by *A. flavus*, *A. niger*, *A. ochraceus* and *A. parasiticus*. The incidence of fungal genera investigated in brown rice were elucidated in Table 2, Fig. 1b. *Aspergillus* and *Penicillium* were the most predominant genera recorded with fungal population lies in the range of 5.4×10^3–3.8×10^6 CFU/g, 2.6×10^3 –1.5×10^5 CFU/g followed by *Fusarium* showed 0.8×10^3 – 0.5×10^5 CFU/g respectively. In polished rice, the findings recorded highest fungal population of 1.8×10^3–0.5×10^6 CFU/g by *Aspergillus* followed by *Penicillium* documented 1.4×10^3–0.9×10^5 CFU/g (Table 3, Fig. 1c).

The study revealed that among the isolated fungal genera, *Aspergillus* spp were the most and predominant fungal contaminant with an isolation frequency of 69.56% followed by *Penicillium* 20.24% and the other genera (10.20%) respectively. In agreement with our findings, Katsurayama *et al*. (2020) documented the *Aspergillus*, *Penicillium* and *Fusarium* were the dominant fungal species found in rice samples marketed in Brazil. Rice in tropical Asia is mostly infected with *Aspergillus* fungi due to the prevailing condition occur during pre-harvest, harvest and postharvest stages (Santos *et al*., 2022). Laut *et al*. (2023) revealed the analysis of rice samples documented the major fungal isolates belong to *Aspergillus* (80%) and *Penicillium* (20%). In India, Begum and Samajpati (2000) isolated *Aspergillus* sp. and *Penicillium* sp. from contaminated rice sold in the local markets of Calcutta. Gautam *et al*. (2012) documented the prevalence of *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia Cladosporium* and *Alternaria* in rice samples.

Morphological characterisation of *Aspergillus niger*. The macroscopic and microscopic characters of *A. niger* isolates were studied on CYA medium (Fig. 2). The colour of the colony was white at initial stage which later turned into brown to dark black due to production of conidial spore. Initially, there was slight yellowish tinge on white mycelia which occurred due to radial cracks in mycelia. Conidial heads are globose, biseriate, dark brown, radiating, and prone to splitting into several loose columns as they age. Conidiophores have smooth walls, hyaline or turn dark towards the vesicle and the conidia are globose to sub globose, black in colour. The morphological characters of *A. niger* was similar with the descriptions given by Klich (2002) reported that the *A. niger* of section *Nigri* characteristically produces dark-brown to black spores. Likewise, Silva *et al*. (2011); Karlton-Senaye *et al*. (2015); Zulkifli and Zakaria 2017; Abdallah *et al*. (2022) documented the growth characteristics of *A. niger* cultured on CYA and MEA (Malt Extract Agar) medium. Diaz *et al*. (2021) reported *A. niger* showed dark brown colour conidia at initial stages on CYA medium which later turns to characteristic black colour. The colour of the colony was dark brown to black on the reverse side of Petri plate. El-Sayed *et al*. (2019) documented the occurrence of *A. niger* in polished rice and found to be OTA producer.

Morphological characterisation of *Aspergillus ochraceus*. The vegetative mycelium of *Aspergillus ochraceus* was white and mostly submerged, while the conidial heads are typically arranged in zones and are closely packed. The colour of the colony was yellow and on the reverse side, pale to brownish in colour (Fig. 3). The colony characters are consistent with the descriptions by Frisvad *et al.* (2004); Visagie *et al.* (2014). By visually under naked eye, conidiophore appear as a powdery mass with chalky yellow colour. The conidial head appeared as globose initially but with age, splitting into two or three divergent columns with smooth wall spherical conidia. Similar colony characters of *A. ochraceus* were documented by Pandit *et al.* (2014) in CYA medium. Abdul-kareem *et al.* (2021) studied the macroscopic and microscopic characters of *A. ochraceus* and reported *A. ochraceus* produced yellow colour colony on PDA medium. Some colonies may form pinkish to purple, pebble-like sclerotia measuring up to 1 mm in diameter and the findings were in line with the report of Borges *et al.* (2011) reported pink to purple sclerotia formation by *A. ochraceus* is the main distinguishable feature for the species.

Species confirmation of *A. ochraceus*. The *A. ochraceus* isolates were subjected to PCR amplification of pks gene using AoLc35F, AoLc35R primer and resulted with the expected amplicon size of 520 bp (Fig. 4). Similarly, Dao *et al*. (2005) used AoLc35F, AoLc35R primer for the specific identification of *A. ochraceus*. Algammal *et al*. (2021) documented the confirmation of *A. ochraceus* isolates was performed by the amplification of polyketide synthase gene (*pks*) using AoLc35F, AoLc35R primer pair. The amplification of pks gene confirmed that the isolates of *A. ochraceus* are ochratoxigenic and involved in the biosynthesis of ochratoxin A. The present findings were in agreement with the report of Moghadam *et al*. (2019) documented the confirmation of *A. ochraceus* as OTA producer using AoLc35F, AoLc35R primer pair.

Table 1: Assessment of fungal contaminants in paddy samples.

Sr. No.	Genus	Range (CFU/g)
1.	*Aspergillus* spp	$8.24 \times 10^3 – 6.03 \times 10^6$
2.	*Penicillium* spp	$4.82 \times 10^3 – 2.76 \times 10^5$
3.	*Fusarium* spp	$1.9 \times 10^3 – 0.7 \times 10^5$
4.	*Rhizopus* spp	$1.62 \times 10^3 – 1.02 \times 10^4$
5.	*Mucor* spp	$1.67 \times 10^3 – 3.8 \times 10^3$
6.	*Alternaria* spp	$2.9 \times 10^3 – 1.4 \times 10^4$
7.	*Curvalaria* spp	$1.91 \times 10^3 – 4.12 \times 10^3$
8.	*Cladosporium* spp	$1.47 \times 10^3 – 0.51 \times 10^4$
9.	*Bipolaris* spp	$0.71 \times 10^3 – 2.54 \times 10^3$
10.	*Trichoderma* spp	$5.1 \times 10^3 – 4.8 \times 10^4$

Table 2: Assessment of fungal contaminants in brown rice.

Sr. No.	Genus	Range (CFU/g)
1.	*Aspergillus* spp	$5.4 \times 10^3 – 3.8 \times 10^6$
2.	*Fusarium* spp	$0.8 \times 10^3 – 0.5 \times 10^5$
3.	*Penicillium* spp	$2.6 \times 10^3 – 1.5 \times 10^5$
4.	*Cladosporium* spp	$0.41 \times 10^3 – 0.77 \times 10^3$
5.	*Bipolaris* spp	$0.64 \times 10^3 – 1.36 \times 10^3$
6.	*Rhizopus* spp	$1.3 \times 10^3 – 0.8 \times 10^4$
7.	*Curvalaria* spp	$0.92 \times 10^3 – 1.61 \times 10^3$
8.	*Mucor* spp	$1.1 \times 10^3 – 2.4 \times 10^3$
9.	*Trichoderma* spp	$0.68 \times 10^3 – 0.83 \times 10^3$
10.	*Alternaria* spp	$0.6 \times 10^3 – 0.4 \times 10^4$

Table 3: Assessment of fungal contaminants in polished rice.

Sr. No.	Genus	Range (CFU/g)
1.	*Aspergillus* spp	$1.8 \times 10^3 – 0.5 \times 10^6$
2.	*Penicillium* spp	$1.4 \times 10^3 – 0.9 \times 10^5$
3.	*Fusarium* spp	$0.5 \times 10^3 – 0.2 \times 10^5$
4.	*Curvalaria* spp	$0.47 \times 10^3 – 0.51 \times 10^3$
5.	*Bipolaris* spp	$0.21 \times 10^3 – 0.45 \times 10^3$
6.	*Mucor* spp	$0.73 \times 10^3 – 0.81 \times 10^3$
7.	*Rhizopus* spp	$1.02 \times 10^3 – 3.98 \times 10^3$
8.	*Trichoderma* spp	$0.29 \times 10^3 – 0.48 \times 10^3$
9.	*Cladosporium* spp	$0.18 \times 10^3 – 0.23 \times 10^3$
10.	*Alternaria* spp	$0.3 \times 10^3 – 0.2 \times 10^4$

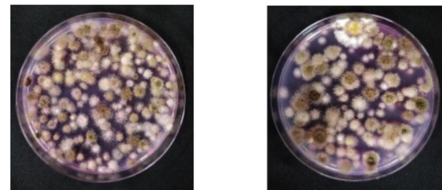


Fig. 1a. Assessment of fungal contaminants in paddy using dilution plate technique.

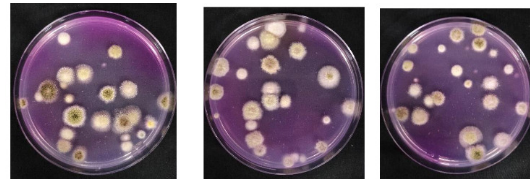


Fig. 1b. Assessment of fungal contaminants in brown rice using dilution plate technique.

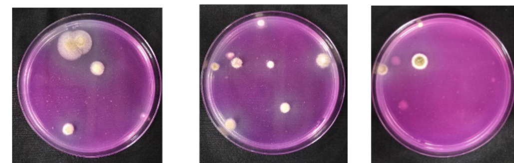


Fig. 1c. Assessment of fungal contaminants in polished rice using dilution plate technique.

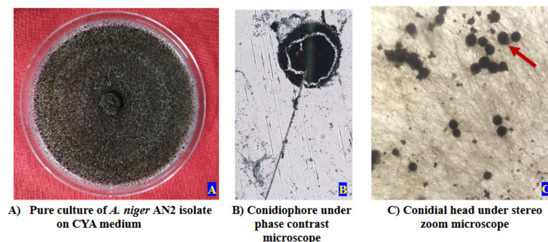


Fig. 2. Morphological characterization of *Aspergillus niger* isolate.

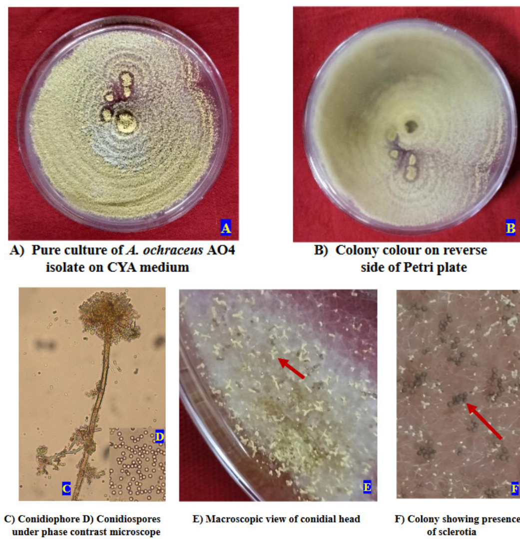
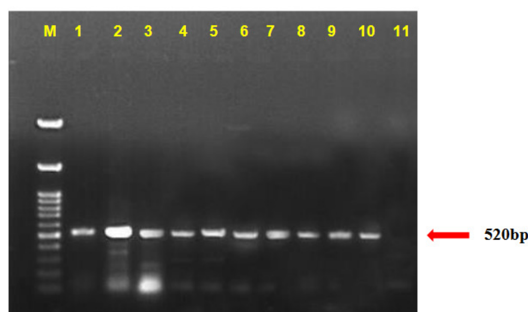


Fig. 3. Morphological characterization of *Aspergillus ochraceus* isolate.



M-100 bp ladder; Lane 1-AO1; Lane 2-AO2; Lane 3-AO3; Lane 4-AO4; Lane 5-AO5; Lane 6-AO6; Lane 7-AO7; Lane 8-AO8; Lane 9-AO9; Lane 10-AO10; Lane 11-Negative control

Fig. 4. Molecular confirmation of *Aspergillusochraceus* isolates by amplification of pks gene using AoLc35F, AoLc35R primers.

CONCLUSIONS

The present findings investigated the enumeration of fungal contaminants in paddy, brown rice and polished rice samples collected from different establishments *viz.,* petty shop, retail and whole sale markets of various districts of Tamil Nadu. The study documented *Aspergillus* spp and *Penicillium* spp as the predominant genera among the collected samples. The ochratoxin A producing fungi such as *Aspergillus niger* and *Aspergillus ochraceus* were identified based on macroscopic and microscopic observation. The colour of the colony on CYA medium was typically brown to dark black in *A. niger* and yellow in *A. ochraceus* with pale to brownish in colour on reverse side. Further, *A. ochraceus* isolates were confirmed molecularly using species specific primer pair AoLc35F, AoLc35R and an amplicon size of 520 bp was obtained.

FUTURE SCOPE

Fungal infection in rice and subsequent ochratoxin A production showcase the importance of adoption of proper agricultural and management practices to prevent and mitigate the fungal contamination. Further, the study emphasis on the need of good storage facilities along with strict monitoring programme for the betterment of safe and healthy food for human livelihood.

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Conflict of Interest. None.

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