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Morphological and molecular identification of *A. flavus* from groundnut soil sample

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Abstract
Aspergillus flavus is the main producer of the well-known carcinogenic aflatoxins. The presence of this fungus and aflatoxins is of huge concern in terms of food safety. *A.flavus* isolated by serial dilution and spread plate techniques. 0.1 ml of 10^{-3}- 10^{-5} dilution sample spread through over the on Potato Dextrose Agar (PDA) plates. The plates were incubated at 28°C for one week, then the fungal colonies were observed and pure cultures were maintained. The identification of fungi at the genus level was carried out by using macroscopic and microscopic examinations depending on the colony color, shape, hyphae, conidia, conidiophores and arrangement of spores. For the molecular identification of the isolated fungi at the species level, the extracted fungal DNA was amplified by PCR using specific internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in Gen-Bank (NCBI). Fungal species were identified as: *Aspergillus flavus.*

Keywords: Groundnut field soil sample, PCR, *Aspergillus flavus*

Introduction
A Fungus is one of the most diverse microorganisms that inhabit different groundnut fields such as soil, water and food sources (Maheswari and Komalavalli, 2013, Sartori *et al.*, 2013; Rebecca *et al*., 2012) [14, 21, 20]. The growth and distribution of fungi are affected by different groundnut factors such as temperature, pH, moisture, degree of aeration, amount and type of nutrients (Gaddeyya *et al*., 2012) [7]. Soil fungi play an important and vital role in maintaining soil fertility and productivity, and are influenced by a number of factors, including soil properties and human activities (Bao *et al*., 2012) [3, 4]. Fungi are very important organisms that inhabit the soil. They play an important part in nutrition and processes that lead to the improvement of the health and development of the plant (Mulani and Turukmane, 2014) [17].

Fungi are high-priority concern because plants are food sources for consumers and are of great economic importance to farmers (Prabakaran *et al.*, 2011) [19]. Udoh *et al*., (2015) [24]. isolated a number of fungi species from groundnut seed that were responsible for post-harvest spoilage of groundnut seed. Molecular identification techniques based on total fungal DNA extraction provide a unique barcode for the determination and identification of fungal isolates up to a species level (Landeweert *et al.*, 2003) [10]. Molecular identification using this barcode has turned into an essential tool for mycologists studying fungal, molecular evolution, population genetics or fungus plant interactions (Moller *et al*., 1992) [15]. The identification of fungi using molecular techniques is carried out by the sequencing of PCR amplified part of 18S RNA genes with universal primers to fungal species (Monod *et al*., 2005; Hensel and Holden, 1996) [9]. On other hand, agriculture is the most important economical resource in this area due to the increase in farming activities over the last two decades. This area contains a large amount of groundwater used for the irrigation of plants. According to the knowledge of the researchers, there is no study dealing with the isolation and molecular identification of fungi from three different districts. This study is aimed at identifying fungi isolated from the soil samples.

Materials and Methods
Collection of Soil Samples
Rhizosphere soil samples were collected from groundnut fields of three districts i.e., Thanjavur, Thiruvarur and Pudukkottai of Tamil Nadu, India. Soil samples were randomly collected in 0-15 cm depth in the near the rhizosphere region of Groundnut field and stored in sterile polythene bags (Akinyanju and Fadayomi, 1989) [1]. The collected soil samples were brought to the laboratory in sterile polythene bags and stored at 4ºC for further analysis.

Isolation fungi
The soil micro fungi were enumerated by soil dilution plate method (Waksman, 1922) [25]. on Potato Dextrose Agar medium. 1gm of soil sample was suspended in 9 ml distilled water to make microbial suspensions from 10^{-2} to 10^{-4}. 0.1 ml of microbial suspension of each concentration was added onto melted, cooled PDA medium supplemented with 1% streptomycin. Inoculated plates were incubated at room temperature 28±2ºC in an inverted position for 5-7 days. Three replicates were maintained for each sample.

Macroscopic and Microscopic Examination of Isolated Fungi
The organism was observed under microscope by using Lacto Phenol Cotton Blue Staining technique. Fungal morphology were studied macroscopically by observing colony features (Texture and Colour) and microscopically by the presence of specific reproductive structures, conidia, arrangements of conidiophores, septate and aseptate mycelium with the help of manual of Gilman (1957); Nagamani *et al.*, (2006) [18].

Molecular Identification of Fungal Species DNA Extraction and PCR Amplification

The DNA Extraction of genomic DNA from the fungi was conducted from a one-week-old PDA culture using DNeasy Plant Mini Kit (Supplied by QIAGEN). Primers (ITS1and ITS4) were used to amplify ribosomal internal transcribed spacer (ITS). PCR products were purified using the QIA quick PCR purification kit (Bao *et al*., 2012) [3, 4].

Sequencing and Analysis

The PCR products were sent for sequencing to Princess Haya Biotechnology Center, Jordan University of Science and Technology. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI) (Liu *et al*., 2000) [11].

Result and Discussion

Macroscopic and Microscopic Features Isolated Fungi

In this study, the isolated fungi were examined on the basis of cultural, microscopic and morphological characteristics (Table-1) show fungal species isolated and identified in this study. The colony morphology of *Aspergillus flavus* shown in Plate-1 reveals a bright yellow-green conidial color, and the microscopic photograph.

Table 1: Morphological Characteristics of Isolated Fungi

S. No	Isolated fungi	Macroscopic view	Microscopic view
1	*Aspergillus flavus*	Green	Conidiophores arise separately from the substratum. 400 -700µ. Chains of conidia few phialides upto 10 – 15 × 3 - 5µ.

Plate 1: *Aspergillums flavus* colony features on PDA and conidia

The Identification of *Aspergillus flavus* Using Molecular Tools

The isolates were confirmed as *Aspergillus flavus* by using specific primers amplifying the DNA fragments of 500bp. The DNA sequence was deposited in NCBI Accession no. MT292809) (Plate 1).

Discussion

This study was carried out to use various morphological and molecular examination methods to identify fungi isolated from the soil from the different District. Fungal species were isolated and identified at the species level using 18S RNA sequences comparison and analysis.

Five of the identified species (*A. Niger*, *R. Stolonifer*, *A.tubingensis*, *A. tenuissima*, *F. oxysporum*) were isolated from the soil. Soil is the most important source for the isolation of fungi, Chandrashekar *et al*. (2014) isolated and identified ten species belonging to three genera (*Aspergillus*, *Penicillium* and *Mucor*) from the rhizosphere soils in different agricultural fields of Nanjangud taluk of the Mysore district, Karnataka, India. The results of another study conducted by Gaddeyya *et al*. (2012) [7]. Revealed the isolation and identification of fifteen species belonging to six genera of fungi from the soil of agricultural fields at Salur Mandal, India. The variation and biodiversity of the isolated fungi from different geographical locations show different factors that affect the growth and distribution of fungi; these factors include soil pH, moisture content, salinity, organic carbon, nitrogen sulfur and potassium (Sharma and Raju, 2013; Yu *et al*., 2007). In this study the fungal isolates were firstly identified to a genus level using a morphological examination depending on the colors of colony formed at sides, the top and reverse of the fungal cultures. The microscopic examination of the shape of the spore-producing structures was used for further identification. The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang *et al*., 2016) [26]. However, this identification is not enough to identify the isolated fungi up to the species level (Lutzoni *et al*., 2004) [12]. The morphological characteristic features of fungal species were observed in the table 1.

The molecular identification was carried out by DNA using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases using NCBI-BLAST. Eight species were identified using DNA with an identity range between 97 – 99 %. It is also proposed that ITS rDNA region sequence is one of the most important tools for the identification of the fungal species isolated from environmental sources (Anderson and Parkin, 2007); hence, it has been widely used to detect the soil fungal community and as an improvement of the classical identifications. ITS rRNA genes are excellent candidates for the phylogenetic analysis because they are universally distributed, functionally constant, sufficiently conserved, and of adequate length to provide a deep view of evolutionary relationships (Madigan *et al*., 2012) [13].

In the present investigation, the isolates were confirmed as *Aspergillus flavus* by using specific primers amplifying the DNA fragments of 500bp. The DNA sequence was deposited in NCBI. (Accession no. MT292809).

According to the results of this study, the biodiversity of the isolated fungal species is clearly observed among the geographical locations. The distribution and abundance of the fungi differ from one isolation location to another such as the *A. flavus* were isolated and identified from the soil. The biodiversity of fungi refers to the physiochemical Properties of the environment such as; pH of the soil, temperature and humidity.

Conclusion

The molecular characterization of *Aspergillus flavus*, were selected on the basis of variation in cultural character and growth pattern. For molecular characterization, ISSR technique is used, were five clusters are reported. The genetic similarity index and genogram support the results.

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