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MOLECULAR IDENTIFICATION OF SEED MYCOFLORA OF OKRA CULTIVARS

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ABSTRACT

Okra (*Abelmoschus esculentus* L. Moench) is the most prominent vegetable crop and extensively grown all over the world for its high nutritive values and year-round cultivation. However, Okra seeds are known to be infected by various seed-borne fungus which act as carrier for the disease outbreak. Seed mycoflora isolated from various okra cultivars were identified by molecular techniques. PCR amplification and sequencing the ITS region of fungal DNA (rDNA) with universal primer pairs ITS1 and ITS4. The sequences of rDNA region were aligned and analysed in the NCBI nucleotide BLAST. It showed 94.54%, 98.75% and 99.26% similarity with the already reported *Fusarium oxysporum* f. sp. *vasinfectum, Aspergillus fischeri* and *A. tamarii* sequences.
Keywords **:** Okra, Fungi, Molecular identification, *Fusarium oxysporum f. sp. vansifectum, Aspergillus fischeri, A. tamarii*

Introduction

Okra (*Abelmoschus esculentus* L. Moench) is a common vegetable crop mainly grown in tropical arid and semi-arid zones in various parts of the world. It belongs to family *Malvaceae*. Okra has a prominent position among vegetables because of its wide adaptability, year-round cultivation, export potential and high nutritive value. In India, highest producer state of okra is Gujarat and in Gujarat main okra growing districts are Surat, Tapi, Navsari, Banaskantha, Dahod, Vadodara, Kheda, Anand, Mehsana etc. Due to high humidity and temperature, different fungi are able to colonize the seed and cause severe crop losses to the farmers.

Okra is known to be affected by different pathogens in the field condition, which are generally carried over by the seeds and disseminated throughout the field during different growing stages. Seed-borne fungal diseases are often the main cause. There are 14 different seed-borne fungal pathogens (Fakir, 2000) causing diseases like seedling blight, stem rot, anthracnose and die-back are considered as major ones (De Tempe, 1964; Neergaard, 1977b; Aulakh *et al.*, 1974; Subbaiah *et al.*, 1982).

Kumar (2019) enumerated the seed-borne microflora associated with okra causing typical disease symptoms viz., Ascochyta blight, pod spots (*Ascochyta abelmoschi*), stem and capsule disease (*Botrytis* sp.), anthracnose (*Colletotrichum dematium*), wilt (*Fusarium oxysporum f. sp. vansifectum* and *Fusarium solani*), charcoal and root rot (*Macrophomina phaseolina* and *Rhizoctonia solani*) etc. and also recorded major fungal genera associated with okra at post-harvest stage namely *Aspergillus flavus, A. niger, A. nidulans, A. fumigatus* etc.

Considering the importance of seed mycoflora of okra cultivars, the present study on various aspects was carried out at the Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand from 2019 to 2021. The experiment for assessment of seed mycoflora of okra cultivars was conducted by using agar plate method. Various mycoflora developed on okra seed samples during seed mycoflora study were cultured separately on PDA and used for the molecular identification. For this purpose,

genetic identification was done of the fungal pathogen and Internal Transcribed Spacer (ITS) rDNA was employed to identify that fungal pathogen at the species level and compared with other similar worldwide fungal isolates available in the NCBI database. Three dominant seed mycoflora i.e., *Fusarium oxysporum* f. sp. *vasinfectum*, *Aspergillus fischeri* and *Aspergillus tamarii* were identified through ITS rDNA sequence technique.

Materials and Methods

Information about workplace

All the research work was carried out in the Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat during the year 2020-21. All the molecular analysis was carried out at the Centre of Excellence in Biotechnology, AAU, Anand.

Identification of Pathogen through Internal Transcribed Spacer (ITS) Region

Among the different DNA marker techniques, the ITS region of fungal DNA (rDNA) has been recognized as the official barcode for fungi (Schoch *et al*., 2012). The ITS region is the widely sequenced DNA region in fungi useful for molecular systematic and has often been used in fungal diversity studies (Nilsson *et al*., 2009). In the present study, DNA barcoding of three seed mycoflora of okra *i.e.*, *Fusarium oxysporum*, *Aspergillus fischeri* and *Aspergillus tamarii* was attempted by targeting the ITS region of fungal DNA (rDNA).

The total genomic DNA was isolated from fungal mycelia using CTAB (Cetyl tri-methyl ammonium bromide) DNA extraction protocol.

The DNA was quantified and qualified using NanoDrop Spectrophotometer and agarose gel electrophoresis, respectively. The samples were stored in deep freeze at -20 °C temperature for long-term usage.

Qualitative Analysis of Extracted Genomic DNA

Separation and visualization of isolated DNA was done on 0.8 per cent agarose.

Chemicals used

- Agarose
- 5X Tris Borate EDTA (TBE) buffer pH 8.0
- Ethidium bromide (10 mg/ml)
- 100 bp ladder

Agarose gel electrophoresis was done for DNA separation. Agarose gel of 0.8 per cent in TBE buffer (1X) was casted in a horizontal gel frame and products were visualized by incorporating 1 µl (10 mg/ml) ethidium bromide per 10 ml of gel solution and viewed in a Gel Documentation System.

Quantification of Extracted Genomic DNA

The amount of DNA in each sample was quantified by taking the readings at 260 nm and 280 nm using NanoDrop Spectrophotometer. The DNA quantity in ng/µl and OD value for each sample was noted. The ratio between the readings at 260/280 nm was used to estimate the purity of the DNA samples.

PCR Conditions

The list of primer, components for PCR reaction showed in Table 1 and 2 (White *et al.* 1990).

Table 1: List of primers

Sr. No.	Primers	Sequence
1	Forward primer ITS 1	5´-TCCGTAGGTGAACCTGCGG-3´
2	Reverse primer ITS 4	5´-TCCTCCGCTTATTGATATGC-3´

Table 2: Components for PCR reaction

Sr. No.	Reagents	Quantity (µl/reaction)
1	DreamTaq green PCR master mix (MBI, Fermentas AG)	5.0
2	Forward primer (10 pM/µl)	0.5
3	Reverse primer (10 pM/µl)	0.5
4	Nuclease free water	2.0
5	Template DNA (50ng/µl)	2.0
Total		**10.0**

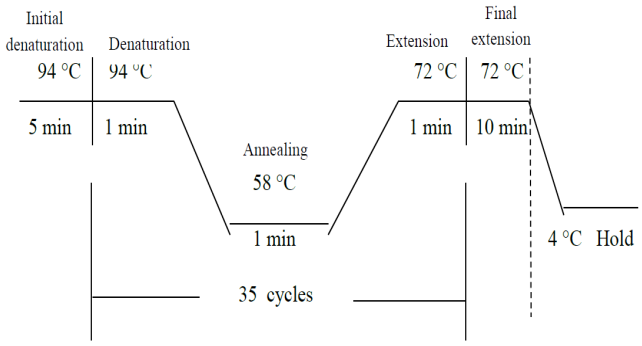


Fig. 1: Programming for PCR (*Fusarium oxysporum* f. sp. *vasinfectum*

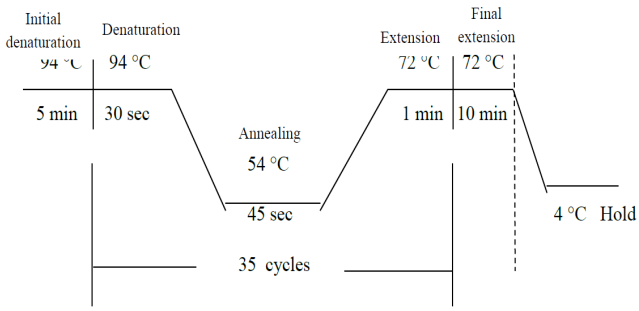


Fig. 2: Programming for PCR (*Aspergillus fischeri*)

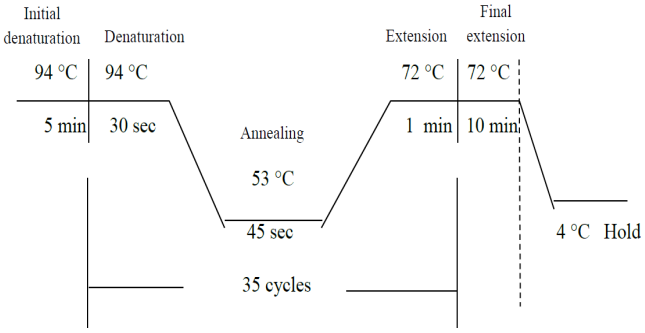


Fig. 3 : Programming for PCR (*Aspergillus tamarii*)

Homology Analysis of the ITS Sequences and Phylogenetic Analysis

Sequences were searched using BLAST (Basic Local Alignment Search Tool) program from the GenBank database of NCBI (National Centre for Biotechnology Information), USA to find out the similarity of newly sequenced samples to the GenBank database.

Result and Discussion

In the present study, DNA barcoding of three seed mycoflora of okra *i.e.*, *Fusarium oxysporum*, *Aspergillus fischeri* and *Aspergillus tamarii* was attempted by targeting the ITS region of fungal DNA (rDNA).

DNA Extraction and Quantification

DNA was isolated using CTAB (Cetyl trimethyl ammonium bromide) DNA extraction protocol as described in materials and methods. The amount of DNA in each sample was quantified using NanoDrop Spectrophotometer by taking the readings at A260/A280 nm and A260/A230 nm (Table 3). Samples with a good concentration of DNA and free from RNA/protein contamination were selected for further analysis.

Polymerase Chain Reaction (PCR)

For PCR amplification, the ITS region of fungi was amplified with universal primer pairs of ITS 1 and ITS 4. The primer combination of ITS 1 and ITS 4 produced an amplicon of 536 bp, 569 bp and 571 bp for *Fusarium oxysporum* f. sp. *vasinfectum*, *Aspergillus fischeri* and *Aspergillus tamarii*, respectively. Amplified products were analyzed on 1.8 percent agarose gel. The representative image of PCR product on agarose gel was depicted in Plate 1.

Table 3: Assay of the DNA samples obtained through NanoDrop Spectrophotometer

Sr. No.	Sample	260/280 nm	260/230 nm	Concentration (ng/µl)
1	Fov1	1.91	1.35	1966.10
2	Af2	1.93	1.80	3450.98
3	At3	1.97	1.58	1045.59

Note: Fov1 = *Fusarium oxysporum* f. sp. *vasinfectum* sample, **Af2** = *Aspergillus fischeri* sample, **At3** = *Aspergillus tamarii* sample

Sequencing Result of Sample

The results were aligned and analysed in the NCBI nucleotide BLAST to find out the similarity of newly sequenced samples to the GenBank databases and matched at the diverse global similarity.

All three ITS rDNA sequences of samples were confirmed as *Fusarium oxysporum* f. sp. *vasinfectum*, *Aspergillus fischeri* and *Aspergillus tamarii* which covered the internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

Fusarium oxysporum f. sp. *vasinfectum* (Accession No. MZ333130)

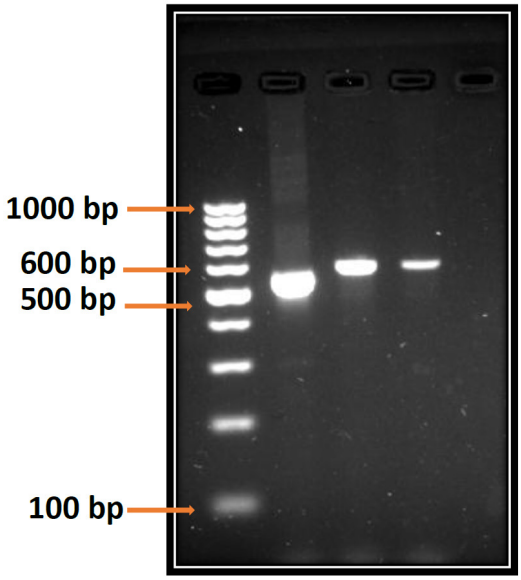
For the ITS rDNA region, BLAST search for similarities of identification showed 94.54% percentage of similarities with the sequences within GenBank. Therefore, the species name assigned was according to the closest BLAST search. The pathogen of interest showed 94.54% similarity with the already reported *Fusarium oxysporum* f. sp. *vasinfectum* (X78259) sequence.

Aspergillus fischeri (Accession No. MZ333227)

For the ITS rDNA region, BLAST search for similarities of identification showed 98.75% percentage of similarities with the sequences within GenBank. Therefore, the species name assigned was according to the closest BLAST search. The pathogen of interest showed 98.75% similarity with the already reported *Aspergillus fischeri* (MH858698) sequence.

Aspergillus tamarii (Accession No. MZ333145)

For the ITS rDNA region, BLAST search for similarities of identification showed 99.26% percentage of similarities with the sequences within GenBank. Therefore, the species name assigned was according to the closest BLAST search. The pathogen of interest showed 99.26% similarity with the already reported *Aspergillus tamarii* (MK165705) sequence.



Gel electrophoresis of DNA amplicon from PCR using primer set ITS1 and ITS4 that amplify (A) 529 bp, (B) 569 bp and (C) 571 bp region of ITS rDNA for *Fusarium oxysporum* f. sp. *vasinfectum, Aspergillus fischeri* and *Aspergillus tamarii*, respectively.

L: 100 bp DNA ladder
A: *Fusarium oxysporum* f. sp. *vasinfectum*
B: *Aspergillus fischeri*
C: *Aspergillus tamarii*

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