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Simple and Rapid Method for Extraction of Good Quality Fungal DNA Directly from Fusarium Wilt Infected Banana and Molecular Characterization of the Pathogen

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A B S T R A C T

The study was undertaken to isolate the DNA of good quality from the banana wilt pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc) directly from infected tissues for molecular studies. In this method, laborious and time-consuming procedures such as isolation of the pathogen and its artificial cultivation are avoided. Moreover, grinding of the fungal mycelium using liquid nitrogen is also not needed as the DNA is directly extracting from infected banana tissues. This is the first attempt to isolate Foc DNA directly from infected tissues. Extraction buffer containing 2 percent CTAB, 0.7 M NaCl, 20 mM EDTA, 50 mM Tris HCl, ß-mercaptoethanol and 1 per cent PVP was used for the isolation of fungal DNA from the infected rhizome. The concentration of DNA was 751.6 ng/µl. OD ratio $\lambda_{260}/\lambda_{280}$ obtained was 1.89 which comesin between 1.8 to 2 indicates the purity of DNA isolated. The ITS region of 5.8S rDNA was amplified using the ITS 1F and ITS 4R primers. A single band of size approximately 580 bp was obtained as a result of PCR analysis. A tree inferred from phylogenetic analysis using Maximum Likelihood method revealed that sequence was closely related with Foc isolates which is supported by a good bootstrap value of 96 percent.

Introduction

Banana is an important fruit crop in tropical and subtropical countries. However, its production is constrained by several diseases that affect yield and quality of the fruits. Fusarium wilt, the most destructive disease of banana was first reported from Panama, Tropical America[1]. Since then, the disease was widely spread throughout the world. In India, Fusarium wilt of banana was first informed from West Bengal in 1911. Then it became the most destructive disease in other banana rising states. In Kerala, the southernmost state of Indian peninsula so far it was reported on banana varieties likes Rasthali, Njalipoovan and Kadali.

Causal agent[2,3] of Fusarium wilt of banana is a soil borne fungus, *Fusarium oxysporum* f. sp. *cubense* (E. F. smith) (Foc)[4]. It is a typical vascular disease and the pathogen enters the

plant through water conducting xylem vessels; thus, causes blockage of water conducting vessels and results in wilted appearance[5]. It is a polycyclic disease; pathogen is capable of causing more than one disease cycle per season[6].

The disease causes huge economic loss to banana farmers. Hence, the proper diagnosis and timely management of the disease are necessary. Cultural and morphological identification of the disease is difficult because of the great diversity among *Fusarium* spp. Nowadays, several studies have been conducted for the identification of *F. oxysporum* at the molecular level using PCR based methods[7].

Isolation of total genomic DNA from Foc infected banana was carried out using CTAB method by several researchers. Extraction buffer consisting of 40 mM EDTA and 100 mM Tris-HCl was used[8] for the isolation of genomic DNA from Foc. Proteins present were removed by the addition of 24:1 chloroform: isoamyl alcohol and the nucleic acid was precipitated by treating with ice cold isopropanol. Molecular characterization ofFoc isolates were carried out with the reaction mixture (25 µl) consisted of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.6 mM of each dNTPs, 0.25 µM of each primer and1.25 U *Taq* DNA polymerase[9]. Molecular characterization of *Fusarium* was carried out to identify the isolates at species level through ITS - rDNA sequence analysis universal primers ITS 1F and ITS 4R[10]. Studies have been conducted to observe the relationship pattern of Foc isolates from banana by means of sequencing and phylogenetic tree analysis[11]. Phylogenetic tree analysis done by Maximum Likelihood method with 1000 bootstraps showed the evolutionary relationship among the Foc isolates[12]. Advanced detection and identification methods could provide more rapid, accurate, and reliable diagnoses of plant diseases at their early stages, which in turn makes it possible to reduce the impact of disease outbreaks[13.]

Materials and Methods

Extraction and gel electrophoresis of fungal genomic DNA

Genomic DNA of the Fusarium wilt infected rhizome collected from Thrissur district of Kerala was extracted for molecular characterization of the pathogen. The tissue was homogenised in 1 ml of extraction buffer consisting of 2 percent CTAB (cetyl trimethylammonium bromide), 0.7M NaCl (sodium chloride), 20mM EDTA (ethylene diamine tetra acetic acid), 50mM Tris HCl, 50 µl of ß-mercaptoethanol and 1 per cent polyvinylpyrrolidone. This mixture was incubated in a water bath at 65°C for 45 minutes.

After incubation, it was treated with equal volume of isoamyl alcohol and centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant obtained was mixed with equal volume of ice-cold isopropanol and kept for 2 hours incubation at -20°C. Again, centrifuged at 13000 rpm for 10 minutes at 4°C. After decanting the supernatant, the DNA pellet was rinsed with 200 µl of each 70 percent and 100 percent ethanol subsequently. The pellet was air dried, dissolved in 50 µl of Tris EDTA buffer and stored at -20°C for further use.

Agarose gel electrophoresis was carried out in Tarson electrophoresis unit to check the quality of the genomic DNA. Agarose gel of 0.8 percent was prepared in distilled water and 1X TAE (Tris-Acetate-EDTA) buffer which provides a source of ions for electric field during electrophoresis. Gel documentation of total DNA obtained was carried out using BIORAD Molecular Imager.

Polymerase chain reaction using ITS primers

Genomic DNA extracted from the infected tissue was quantified using the instrument NanoDrop spectrophotometer (NanoDrop ND-1000) based on absorbance readings at 260 and 280 nm (λ_{260}/ λ_{280} ratio).

PCR amplification reactions were carried out in 25µl reaction mixture in a Nexus gradient Master cycler. Each reaction mixture consisting of 10X PCR buffer, 25mM $MgCl_2$, 0.2 mM dNTP, DNA sample, 10 pmol of forward primer and reverse primer, *Taq* DNA polymerase and distilled water.

The ITS region of rDNA was amplified using the universal primers, ITS 1F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4R (5'-TCC TCC GCT TAT TGA TAT GC-3'). PCR amplification profile followed was as follows; initial denaturation at 94°C for 10 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 54.9°C for 1 minute and extension step at 72°C for 1 minute, followed by final extension at 72°C for 10 minutes.

The PCR products were resolved in 1.5 percent agarose gel stained with ethidium bromide (10mg/ml) immersed in 1X TAE buffer and visualized.

Sequencing and phylogenetic analysis

The sequencing of the amplified product obtained through PCR using universal primers ITS 1F and ITS 4R was done on Sanger platform. BLASTn of the sequence revealed the identity of the pathogen and sequence was submitted to GenBank.

The sequences of *Fusarium* spp. selected from NCBI along with the sequence of isolated DNA were aligned with ClustalW tool accessible in MEGA-X software by means of Maximum Likelihood construction method with a bootstrap value of 1000.

Results and Discussion

Isolation of DNA

The isolation of good quality DNA of the pathogen is required for molecular works and is important for the proper management of the disease. The method employed here is rapid and inexpensive which avoids the time consuming and difficult process explained in former methods as the isolation of pathogen[14], its artificial cultivation and grinding with liquid nitrogen[15].

Extraction buffer used in this protocol was the modifications of earlier reported method for extraction of DNA from Foc culture[16]. In this protocol, 2 per cent CTAB and 20 mM EDTA were added instead of 1 per cent CTAB and 25 mM EDTA used in former method.

In addition to this, 50 µl of ß-mercaptoethanol and 1 per cent polyvinylpyrrolidone were also used during the grinding of 1 mg tissue. EDTA inactivates[17] the endonucleases and ß-mercaptoethanol prevents the phenolic oxidation.

CTAB avoids the polysaccharides contamination whereas, the isopropanol and ethanol allow the actual precipitation of DNA and remove the salts and detergents, making it more stable. The quantity of DNA extracted was measured using a NanoDrop spectrophotometer. The concentration of DNA yield was 751.6 ng/µl. OD ratio λ_{260}/ λ_{280} obtained was 1.89 which relies between 1.8 to 2 indicates the DNA is free from protein and RNA impurities[18]. Gel electrophoresis of the isolated DNA was carried out and documented using BIORAD Molecular Imager (Figure.1).

Amplification of ITS – rDNA region

A range of temperature from 54.7°C to 57.7°C was used for the standardization of annealing temperature and the good quality band of amplicons were obtained at 54.9°C.

The isolate was amplified at 580 bp using ITS 1F and ITS 4R primers[19] andPCR product obtained was compared with GeNei™ StepUp™ 100 bp DNA ladder[20.] Figure 2 represents the amplification of PCR product in 1.5 percent agarose gel.

Sequencing and phylogenetic analysis

The sequencing[21] of amplicon obtained through PCR was done and compared with top hit accessions available in NCBI database. *In silico* analysis showed that the isolate exhibited 100 percent similarity with *F. oxysporum* f. sp. *cubense* accessions infecting banana. The sequence was submitted in NCBI database through BankIt program[22] and obtained the accession number MN953004.

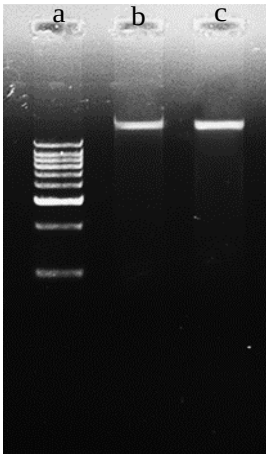

Figure.1 Documentation of DNA isolated. A) 1 kb molecular marker. b & c) replica of Foc DNA isolated

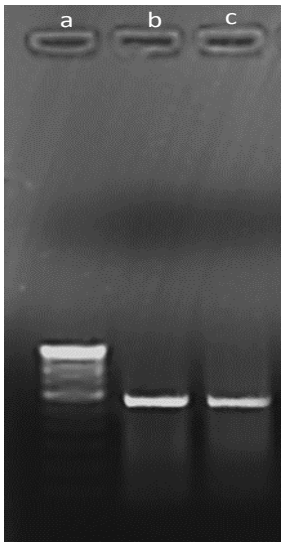

Figure.2 PCR product amplified with ITS 1F and ITS 4R primers a) 100 bp molecular marker. b & c) replica of PCR products of Foc isolate

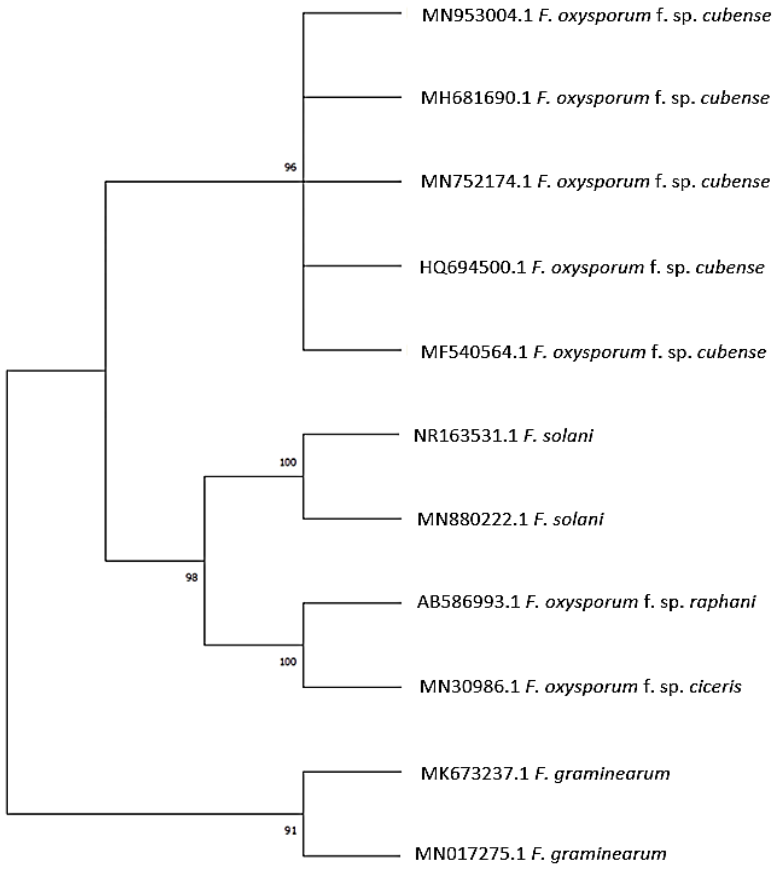

Figure.3 Phylogenetic analysis of Foc isolate MN953004 on the basis of ITS primers, dendrogram generated using MEGA X software with Maximum Likelihood method

The phylogenetic analysis of the isolate MN953004 was done by aligning the sequences of various *Fusarium* spp. available in NCBI. The phylogenetic tree constructed by Maximum Likelihood method[23] with a bootstrap value of 1000using MEGA X software[24]was shown in the Figure3. The sequences were classified into two major clades.

The first clade included all *F. oxysporum* and *F. solani* isolates whereas, second clade consisted of *F. graminearum* isolates. The isolate MN953004 was clustered in the first clade along with four other Foc isolates taken from NCBI. The phylogenetic tree analysis shows that the isolate MN953004 belongs to the Foc group with 96 percent of bootstrap support.

In conclusion, Good quality DNA of *Fusarium oxysporum* f. sp. *Cubense* (E. F. Smith) can be isolated using extraction buffer containing 2 percent CTAB, 0.7M NaCl, 20 mM EDTA, 50 mM Tris HCl, 50 µl of ß-mercaptoethanol and 1 per cent PVP. Foc gives better amplification at an annealing temperature of 54.9°C and a band size of approximately 580 bp was obtained. The isolate used for the study was sequenced and *In silico* analysis displayed 100 percent similarity with Foc accessions. The sequence was deposited in NCBI database and the accession number was obtained as

MN953004. The phylogenetic tree clearly showed that the isolate MN953004 was closely related with Foc isolates with a good bootstrap value. This method can be used for isolation of high-quality DNA required for molecular research.

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