Original Research Article

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Molecular detection of *Fusarium oxysporum* f. sp. *cubense* Race TR4 infecting banana groups in Indo-Gangetic plains of India

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~~**Molecular Detection of Tropical Race 4 of *Fusarium oxysporum* f. sp. *cubense* on Cavendish (AAA) Group of Banana in Indo-Gangetic plains of India**~~

Abstract

Panama wilt or Fusarium wilt incited by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a major threat for banana cultivation and impacting symbolic economic losses worldwide. Previously *Foc* race 1 and race 2 was detected in India which spread monoculture of Cavendish bananas resistant against these two races. Later on, another new race TR4 of *Foc* was detected to be incited in 'Cavendish' group of banana raised an alarming situation to the global banana industry. In the present study detailed survey was conducted in banana growing districts of West Bengal, India for visualizing the incidence of disease as well as collection of isolates from infected suckers and rhizomes of '~~Cavandish'~~ Cavendish' banana ~~–~~with an aim to develop a rapid detection technique for ~~identification~~ detection of the presence of *Foc* TR4 race at early infection stages. Field survey was conducted in popular banana growing districts of West Bengal, India followed by isolation of pathogen, test of ~~pathogenecity~~pathogenicity and further molecular detection through *Foc* TR4 specific primers. Results revealed that the colonies formed ~~in~~ on the potato dextrose agar (PDA) medium exhibited typical characteristic of *Foc*. Pathogenicity test conducted by considering healthy plantlet of Grand Naine also detected characteristic symptom of *Foc*. Additionally, PCR assay using specific markers followed by sequencing of 28S-18S ribosomal RNA IGS confirmed the presence of this isolate in the infected samples. Phylogenetic evolution assays revealed a very close relationship of the *Foc* TR4 strains of India with Asia-Pacific isolates of *Foc* TR4. This study

advocated the urgency of prevention of introduction of *Foc* TR4 into disease-free areas for maintaining sustainable banana production.

Keywords: Banana, Cavendish (AAA), *Fusarium oxysporum* f. sp. *cubense*, Tropical race 4, and PCR

1. INTRODUCTION

Bananas or Plantains (*Musa* spp.) is are the important fruit crops in the World and considers as a source of nutrition and livelihood of many developing countries in the tropics. This fruit crop is considered as a cheap source of nutrition to the economically poor vulnerable sector of the society with having export potentiality (Ref???). Despite of having immense potential, it remains undervalued and suffers from many biotic and abiotic constraints. Among the biotic stresses, Fusarium wilt or Panama disease of banana, incited by *Fusarium oxysporum* f. sp. *cubense* (Foc), is the most fatal plant disease [1,2]. Originating in Asian region, this deadly disease disseminated into Africa and America at an alarming pace and affected most of the commercially exported banana cultivars [1,3-,4].

The first visible symptom of Fusarium wilt in banana is classified into two types of external symptoms: “yellow leaf syndrome” and “green leaf syndrome” [1,4]. In the infected plants yellowing and wilting of older leaves are observed followed by wilting of the younger leaves and complete defoliation and dying of the infected plants. Internally, in the infected plants reddish brown discoloration is observed in the xylome with necrosis in the pseudostem [1,2,5]. (Foc is a soil borne pathogen and intricate to manage due to absence of effective physical, chemical and biological control measure [6]. Therefore, identification of resistant sources is the only effective way to resolve the issue. Based on pathogenicity test, previous studies have reported banana cvs. Manzano (AAB) and Gros Michel (AAA) as differential host for race 1 whereas, cv. Bluggoe (ABB) for race 2 [4,7]. This resulted complete removal of the susceptible cultivars and rapid cultivation of resistant Cavendish cultivars against Foc

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Race 1 which are currently the source of 99% of banana exports. Unfortunately, later on another race was identified in both tropical and subtropical region and designated as subtropical race 4 (ST4) and tropical race 4 (TR4) and caused great devastation and economic losses in banana export industry based on cv. Cavendish [8-~~11~~,9,10,11]. Race TR4 has resulted in catastrophe to 'Cavendish' banana production in Malaysia, Indonesia, South China, Philippines and Northern parts of Australia [2,12,13]. Later on it became major threat in Australasia afflicted 100,000 hectares of area keeping scope of further dissemination in new areas in more endemic form due to rapid expansion of area under 'Cavendish' banana [14]. Co-evaluation of different isolates and their close proximity create ambiguities in genetic characterization of the isolates thus seek immediate attention for effective characterization that could be of significance in management of these volatile races.

India is the largest producer of banana in the world with an annual production of 29.163 million tones [15] and is considered as one of the ~~centre~~center of diversity of banana. More than 20 banana varieties are grown commercially in the country and more than half of India's banana belongs to 'Cavendish' group. Panama wilt was first identified in India from the state of West Bengal in 1911 [16]. Recently, the incidence of VCG 0124 of race 1 on 'Cavendish' group of banana was also reported from other states of India [17,18].

Identification of diseased plants is difficult in early stages of infection as the infected suckers and rhizomes are devoid of symptoms until four months old. Therefore, symptomless infected suckers can easily spread the fungus to new area and may cause epidemic. Therefore, early detection and prevention of further spread of the disease is imperative for management of this aggressive strain. Keeping these in the backdrop the aim of this present investigation was to develop a rapid detection technique to identify Foc TR4 in early infection stages in the infected 'Cavendish' banana suckers and rhizomes which will be helpful to import disease free suckers and restrict further movement of the disease to other parts of India.

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2. MATERIALS AND METHODS

2.1. Field survey of wilt infected banana rhizome

A detailed survey work was conducted in 10 different locations of 3 commercial banana growing districts of West Bengal *viz*. Nadia, 24 Parganas (North) and Hooghly. The investigation was conducted during 3 seasons of 2017-18 *viz*. summer, rainy and winter on different commercial cultivars including 'Rasthali' (AAB), 'Cavendish' (AAA), 'Karpuravalli' (ABB) and cooking varieties (ABB) based on the external and internal symptoms. Interestingly, among the different cultivars only the 'Cavendish' banana group (Grand Naine and Robusta) was identified with the typical panama wilt symptom of variable degrees in two villages of Nadia district namely, Jallalkhali ("23.34 N, 88.51 E") and Dogachi ("23.20 N, 88.30 E"), in Krishnanagar, West Bengal (Fig. 1, 2 and 3). The percent disease incidence was calculated by recording number of wilted plants from four sides of each filed and ~~centre~~center region of the two villages using the following standard formula.

~~Percent~~ % Disease Incidence = (Number of wilted plants/ Total number of plants observed) × 100

2.2. Sample collection and Isolation of pathogen

Samples in the form of different portion of pseudostems were collected time to time from diseased fields from the infected plants. Sections of pseudostems were thoroughly washed with running water and cut into 5 cubic mm pieces and were surface sterilized with freshly prepared 0.1 % $HgCl_2$ solution. The surface sterilized pseudostem pieces were further transferred to PDA media amended with Streptomycin sulphate [19]. Colonies confirmed with the presence of macroconidia, microconidia and chlamydospore having the typical characteristic of Foc under light microscope (Zeiss; Axio Lab.A1) and were transferred

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ascetically on potato- dextrose agar (PDA) media to isolate pure culture of the pathogen (Fig. 4). The cultures were stored after purification ~~for future~~ untill use at 4°C.

2.3. Pathogenicity Test

Three months-old suckers of healthy Grand Naine collected from ~~All~~ all India Co-ordinated Research Project (AICRP) on Fruits, Bidhan Chandra Krishi Viswavidyalaya, West Bengal, India were used as test plants for conducting the pathogenicity test. The healthy rhizomes of Grand Naine were planted in large cement pots, filled with 10 kg sterilized potting mix (1:1:1 ratio of garden soil, sand and decomposed farmyard manure, w:w:w) under poly house conditions. 30 days old banana plants were inoculated by root dipping for 30 minutes in conidial suspension (10^6 conidia-/-mlL) of Foc isolates S1, S2 and S2a [20]. The inoculated test plants along with the uninoculated control plant were maintained at 28°C and 80 % relative humidity conditions inside greenhouse. Disease incidence was recorded at every 7 days interval post inoculation. The recommended scale was used to evaluate the extent of external symptoms produced, wherein 1;-: depicted no symptoms, 2;: depicted initial yellowing in lower leaves, 3: indicated yellowing of old lower leaves accompanied with discolouration of the younger top leaves, 4;-: depicted intense yellowing of all leaves and 5;-: depicted dead plant [19]. PDA medium was used for isolation, purification and verification based on colony and conidial characters (Fig. 5).

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2.4. DNA Extraction, Purification and PCR amplification

DNA was extracted from ~~5~~ five days-old Potato Dextrose broth culture of the morphologically confirmed *Foc* isolates. The fungal biomass mat was harvested by decanting the liquid broth. Mycelia mat (1.5 cm) weighing up to 20 mg was cut with sterile blade and put into Eppendorf tubes. Total genomic DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega) following manufacturer's protocol. DNA was collected

in 200 ~~µl~~ µL molecular grade distilled water and stored at -20°C with proper labeling. A total of 25 ~~µl~~ µL PCR reaction was set up which contained 4.0 ~~µl~~ µL of DNA (Concen…??), 2.5 ~~µl~~ µL of 10X PCR buffer, 1.0 ~~µl~~ µL each of 20 pm/m~~l~~L forward and reverse primer, 1.5 ~~µl~~ µL of 25 mM $MgCl_2$ (Concent. Is not enough??, it should be 2.5 µL of 50 mM $MgCl_2$), 0.5 ~~µl~~ µL of 10 mM dNTPs mix, 0.5 µlL of 1 U *Taq* polymerase (It should be 1.0 µL) and 14 (Revise??) ~~µl~~ µL of double distilled sterile water. PCR amplification was carried out using *Foc* TR4 specific primer pair 01213/16 F1 (5′-ACGTTTAAGGTGCCATGAGAG-3′) and 01213/16 R2 (5′-CCTCGTGAGCCACTTTTTAT-3′) [21]. The amplification reaction was carried out in a ~~thermocycler~~ Thermocycler (Name of machine, code..?) with the following parameters: initial denaturation at 94°C for 2 minutes (Is not enough, should be 3-5 min)???; 35 cycles consisting 45 sec of denaturation at 94°C, 45 sec of primer annealing at 63°C, 60 sec of extension at 72°C; and a final extension for 10 min at 72°C. Amplified PCR product were separated by electrophoresis on 1.5% agarose gel prepared in 1X TAE buffer, added with ethidium bromide. Visualisation of the gel electrophoresis was done using BIO-RAD Gel Documentation system with the help of 1 kb DNA ladder (Fermentas, Life Science) (Fig. 6, It is not in the right place, transfer to result) . The amplified products were stored in -20°C for further ~~use~~study. The genomic DNA was further subjected to amplification of ITS region using ITS1 and ITS4 primer pair. The annealing temperature for ITS amplification was set at 55 °C.

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2.5. Sequencing and Phylogenetic analysis

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All the PCR amplified products (Should be purified before sequencing) were sequenced utilizing commercial sequence facility of Agri Genome Labs Pvt Ltd. (Hyderabad). The sequencing was done using ABI 3730Xl DNA Analyser. The complete nucleotide sequences were subjected for similarity search using BLASTn search program (http://www.ncbi.nlm.nih.gov/BLAST/). All the retrieved sequences were submitted to

GenBank with legal annotations. The accessions of GenBank were also received from Who??? And Why ??? (Accession numbers: MH454071, MH454072, MH454073, MH457246 and MH457247). The sequences were compared with equivalent sequences from a range of other Foc race TR4 isolates ~~present~~ documented in GenBank. Multiple sequence alignment was carried out using the software ClustalW and ClustalX version 2.0 [22]. The evolutionary history was inferred using the Neighbor-Joining method [23] and further Phylogenetic trees were constructed (Fig. 7 and 8). The optimal tree with the sum of branch length = 18.50000000 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) ~~were~~was shown next to the branches [24]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [25] and are in the units of the number of base differences per sequence. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 413 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [26].

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3. RESULTS AND DISCUSSION

3.1. Survey, Disease Incidence and Symptomatology

Detection of Foc TR4 race in the Cavandish group of bananas in Taiwan in the year 1994 followed by its transmission in other banana growing countries considered as a serious threat in the export industry and economy of those countries. India was not an exception where this race was probably introduced through disease infected planting materials from its neighboring countries where Foc TR4 was detected earlier. In India, Foc TR4 incursion was not reported as a regular phenomenon and recently it has been identified from the Katihar district of Bihar and from Uttar Pradesh also [27,28].

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In the present study survey has been conducted in the three important banana growing districts of West Bengal during winter season (December, 2018). However, disease was ~~deteered~~detected in Jallalkhali ("23.34 N, 88.51 E") and Dogachi ("23.20 N, 88.30 E"), villages of West Bengal, India,. Grand Naine and Robusta (Cavendish group) exhibited symptoms like chlorosis and yellowing of old leaves with petiole buckling, splitting of pseudostem at the base and reddish brown to black vascular discoloration with no visual symptoms in fruits. Interestingly, no visible symptoms were observed in 3-5 months--old cut open rhizome or suckers. In both the locations, high disease incidence was observed where, 72.5% of disease incidence was observed in Jallakhali and 75% incidence was detected in Dogachi village. Symptoms observed in the present study over the infected Cavendish group of banana were similar to the earlier report [6]. Jallakhali and Dogachi villages are the belt of banana cultivation in Nadia district of West Bengal where monoculture of banana cultivation is the only practice followed since long back. Farmers of these areas were unaware about the bio-safety related issues and instead of using healthy planting material, suckers from infected plant are commonly used as propagating material. This might be the reason behind wide outburst of the disease in these villages.

3.2. Isolation and morphological identification of *Foc* isolate

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Within 3-5 days of transfer of infected pseudostem in the PDA media, white and pink aerial mycelial growth was observed over the pseudostem pieces. Radial growth of the isolates was completed 9 days after incubation. The initial growth of the isolates in the media was thin followed by vigorous fluffy growth showing violet colour pigmentation. Plenty of straight to falcate shaped and two to three septate macroconidia (25 - 40 x 3.6 - 4 µm in size) having boot shaped foot cells were also observed. Elliptical and kidney shaped aseptate micro conidia (6.64-10.45 x 2.24-3.50 µm in size) were abundantly produced. Chlamydospores were produced abundantly either singly or in chains (Fig. 4). In the present investigation, the

microscopic studies carried out with the violet colour pigmented culture of the Foc isolates obtained from infected Cavendish bananas revealed resemblance of the asexual structures studied earlier [19]. The violet color pigmented culture was also affirmed by the previous studies as the typical characteristic of Foc in PDA medium [1,3,29]. Thus, present study competently proved the morphological identity of the isolates from the infected banana plants as Foc.

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3.3. Pathogenicity testing

Pathogenecity test is the prerequisite for determining the formae of the isolates as parasitic isolates of *F. oxysporum* could not be differentiated from the saprophytes [30]. In the present study, all the Foc isolates (Foc- S1, Foc - S2 and Foc – S2a) (Table 1) produced typical symptom of Panama wilt to both Grand Naine and Robosta cultivars during pathogenicity testing at greenhouse**.** It was observed that 7 days after inoculation, the lower leaves started turning yellow followed by shedding of the infected leaves with little discoloration of younger leaves. Disease severity could be categorized in to scale ~~3~~ three as per the earlier classification [19] based on the external symptoms in the infected plants of both the cultivars. Further, culture was raised from the re-isolated pathogen from artificially inoculated wilt infected banana plants resembled typical colony characteristics and conidial morphology of *Foc* isolates as observed during initial isolation from survey (Fig. 5). In the present study, pathogenicity test conducted with the Foc isolates produced external symptoms on the inoculated Cavendish test plants were identical with the observations reported earlier [20] and matched with the scale 3 of external symptom evaluation [19]. ~~Pathogenecity~~Pathogenicity test confirmed that the *Foc* isolated from the Cavendish banana plantations of Jallakhali and Dogachi was TR4 race.

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3.4. PCR detection of the FocTR4 isolate

Detection of pathogen is an important consideration and conventionally, ~~pathogenicity~~pathogenicity test and Vegetative compatibility grouping (VCG) are deployed which having some bottleneck [31,32]. Recently PCR based detection method have been considered as reliable protocol for early and accurate detection of the isolate identity followed by apprehension of diversity and species synteny [20,27,33]. In the present study high quality genomic DNA was obtained from the Foc infected Cavendish cultivars (Grand Naine & Robusta) after morphological confirmation in Potato Dextrose broth culture. PCR amplification using the TR4 (VCG 01213/16) specific primer pair resulted expected amplicon of 455 bp (Fig. 6). PCR with ITS1/ITS4 primer set amplified expected band of approximately 600 bp from DNA extracted from the cultures of Foc isolated from both Cavendish cultivars (Grand Naine and Robusta) that consisted of both ITS region and 5.8S regions. The sequences retrieved were further analysed through NCBI blast. PCR with the template DNA using TR4 (VCG 01213/16) specific primers finally proved the Foc isolates of West Bengal collected from Cavendish group of Banana (Grand Naine and Robusta) was TR4 with the generation of 455 bp amplicon consisting the partial conserved sequence of 28S-18S ribosomal RNA intergenic spacer region (IGS). The primer pairs were designed earlier [21] by utilizing the SNP identified from the IGS region and successfully detected TR4 (VCG 01213/16) among other races and vegetative compatibility groups of Foc by amplifying 455 bp region of the IGS. Similarly, these pair of primers was used previously to find out the distribution of *Foc* TR4 in Asia and reported amplification of 455 bp amplicon only in cases of VCG 1213/16 [34]. Confirmation of the presence of TR4 isolate in Pakistan through deploying IGS specific primer of TR4 was also reported [33].

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3.5. Sequencing and phylogenetic analysis

The partial nucleotide sequences of Foc TR4 infecting Cavendish banana cultivars (Grand Naine and Robusta) were deposited in the GenBank and assigned with accession numbers of

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MH454071 (592 bp), MH454072 (588 bp), MH454073 (589 bp), MH457246 (455 bp), and MH457247(427 bp). Blast analysis of the sequences of ITS region (MH454071, MH454072 and MH454073) depicted 99.64, 99.30 and 99.73% nucleotide sequence identity (nsi), respectively, with Foc tropical race 4 strain B2 rDNA repeat region (LT571434, Netherland). Whereas, ~~Blast~~ blast analysis of the sequences of IGS region (MH457246 and MH457247) revealed 99.32 and 99.53% nucleotide sequence identity (nsi), respectively, with Foc voucher BRIP 62353a 28S ribosomal RNA gene and 28S-18S ribosomal RNA intergenic spacer, partial sequence (KX101211, Australia) and Foc race 4 strain B2 rDNA repeat region (LT571434, Netherland). Multiple sequence alignment was carried out using ClustalW and ClustalX version 2.0 affirmed the same result as mentioned above.

Further, phylogenetic tree was drawn to analyze the phylogenetic relationship of the sequenced genome of Foc TR4 isolates with a number of selected Foc TR4 DNA sequences retrieved from GenBank. Phylogenetic tree was constructed using ITS and IGS region separately. Both the phylogenetic tree clearly indicated that Foc West Bengal isolates S1, S2 and S2a were most closely related with the Foc TR4 isolates of Asia (Fig. 7 and 8). The introgression of Foc TR4 isolate in West Bengal was not having any well established hypothesis. In the present study, the evolutionary relationship drawn with the sequence of amplicon generated from amplifying IGS and ITS region using MEGA 7 revealed close phylogenetic relationship of the West Bengal isolates with isolates from Karnataka, Uttar Pradesh and Bihar in India and other Asian isolates from Taiwan, China, Indonesia and Pakistan [14,21,27,28,35,36].

4. Conclusion

Disease incidence and symptomatology of the surveyed districts confirmed the prevalence of panama disease in 'Cavandish' group of banana. ~~Pathogenecity~~Pathogenicity testing further established the typical symptom of Panama wilt to both Grand Naine and Robosta cultivars.

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Molecular detection as well as phylogenetic study using both ITS and IGS region validated that the Foc West Bengal isolates were closely related with the Foc TR4 isolates of Asia. Hence, it is the first molecular evidence for the presence of Foc TR4 from Indo- Gangetic plains. As the disease goes undetected in 4 months young rhizomes, the present report highlighted the urgency of extending molecular detection through deploying Foc TR4 specific primer set for proper diagnosis of the disease in the infected rhizomes in nursery and in quarantine stations. Rapid identification and eradication of the *Foc* TR4 infected rhizomes is the only way to curtail down the spread of this devastating race of *Foc* within the country and protect the Cavendish banana plantation of India.

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Table 1: Details of the *Foc* isolates collected from West Bengal

~~S.~~ No	Isolates	Cultivars of the isolates	Locations
1	S1	Robusta (AAA)	Dogachia, Krishnanagar
2	S2	Grand Naine (AAA)	Jallalkhali, Krishnanagar
3	S2a	Tissue cultured Grand Naine (AAA)	Jallalkhali, Krishnanagar

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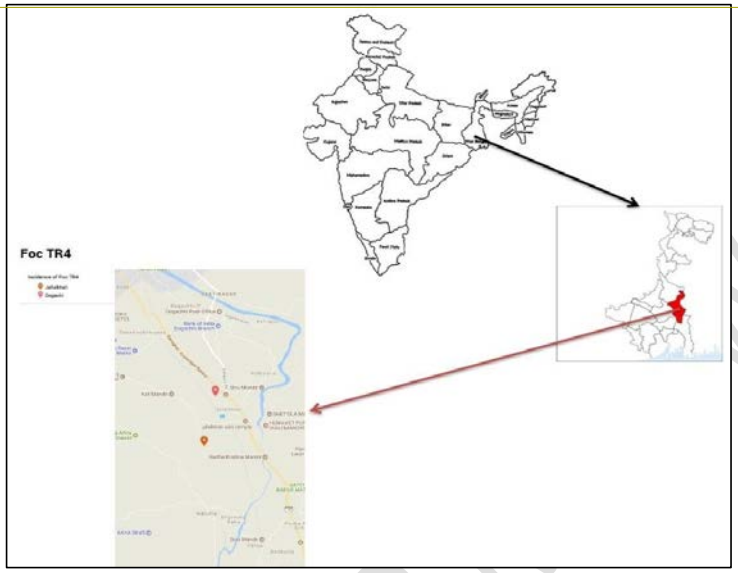

Fig. 1 Map showing incidence of Foc TR4

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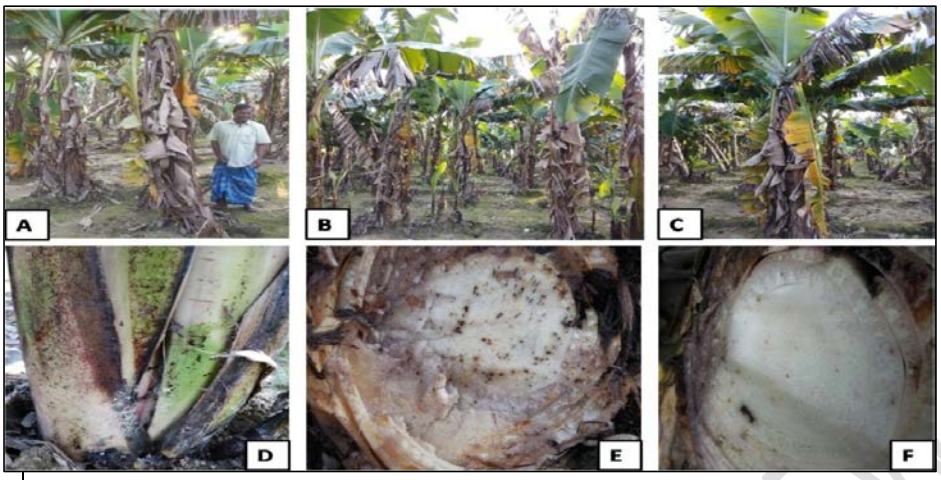

Fig. 2. Cavendish group of banana cultivar (Rubusta-AAA) highly infected with Panama wilt in Dogachia, Krishnanagar, West Bengal; (A-B): Banana field infected with Pnama wilt, (C): Symptoms of Banana plant showing yellowing of leaves with petiole buckling, (D): Splitting of pseudostem, (E): internal symptom with vascular discoloration, (F): No discoloration present (in healthy plant).

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Fig. 3 Cavendish group of banana (Grand Naine cultivar -AAA) highly infected with Panama wilt in Jallakhali village of Krishnanagar, West Bengal;(A-B): Symptoms of banana plant showing yellowing of leaves with petiole buckling, (C): Splitting of pseudostem, (D): internal symptom with vascular discoloration, (E): No discoloration present (in healthy plant).

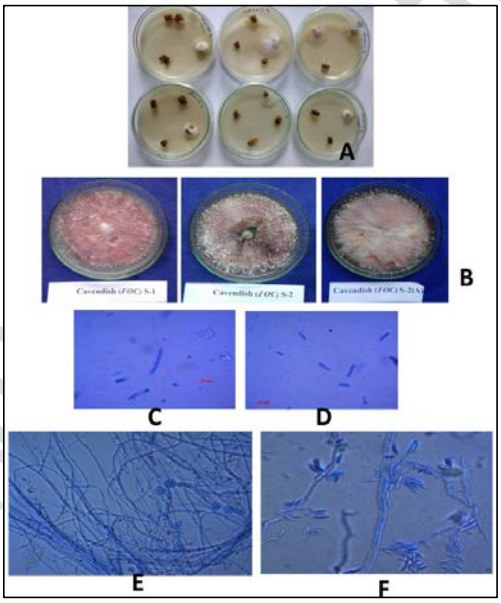

Fig. 4 A. Isolation from 5 cubic mm pieces of infected pseudostem. B. Pure culture of Foc in PDA media. C. Macroconidia (100 x) D. Microconidia (100X) E. Chlamydospore. F. Hyphae containing macro and micrconidia.

(Please write the letter on photos, and divide them into two photos, 1st cultural , 2nd morphology….)

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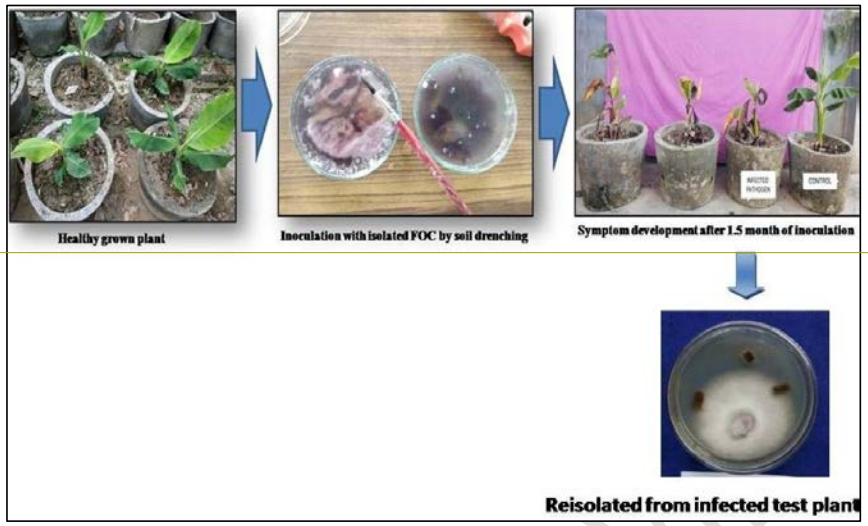

Fig. 5 Pathogenicity test using Cavendish banana as test plants. Rearrange the photos into two lines

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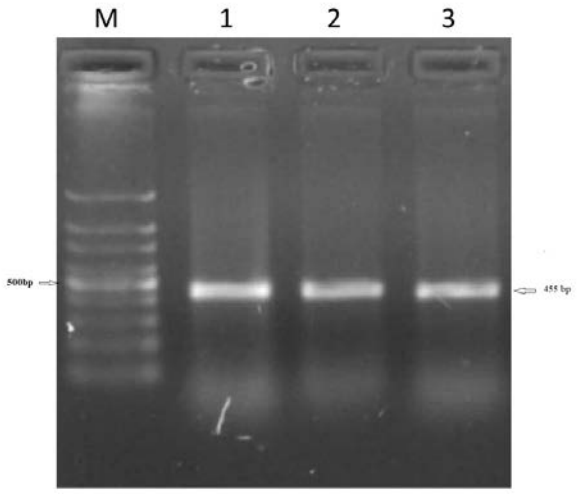

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~~F~~ig. 6 Lane 1 represents 100 bp ladder and 2, 3,4 represents 455 bp amplicon generated by using primer pair *Foc* TR4 01213/16 F1 and 01213/16 R2. (Rewrite the description again, it is not good?????)

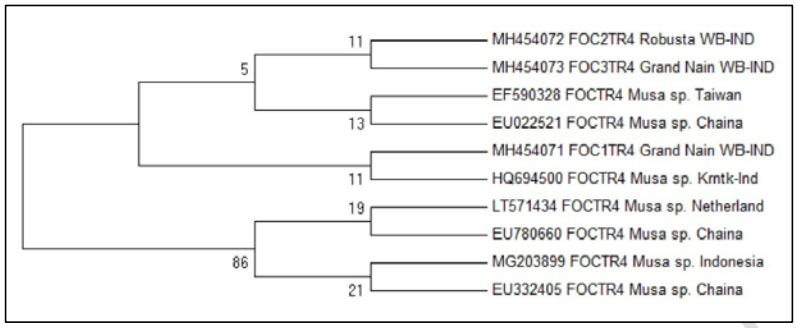

Fig.7 Molecular Phylogenetic analysis using nucleotide sequences amplified using ITS primer pair, the evolutionary history was inferred using the Neighbor-Joining method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Evolutionary analyses were conducted in MEGA7.

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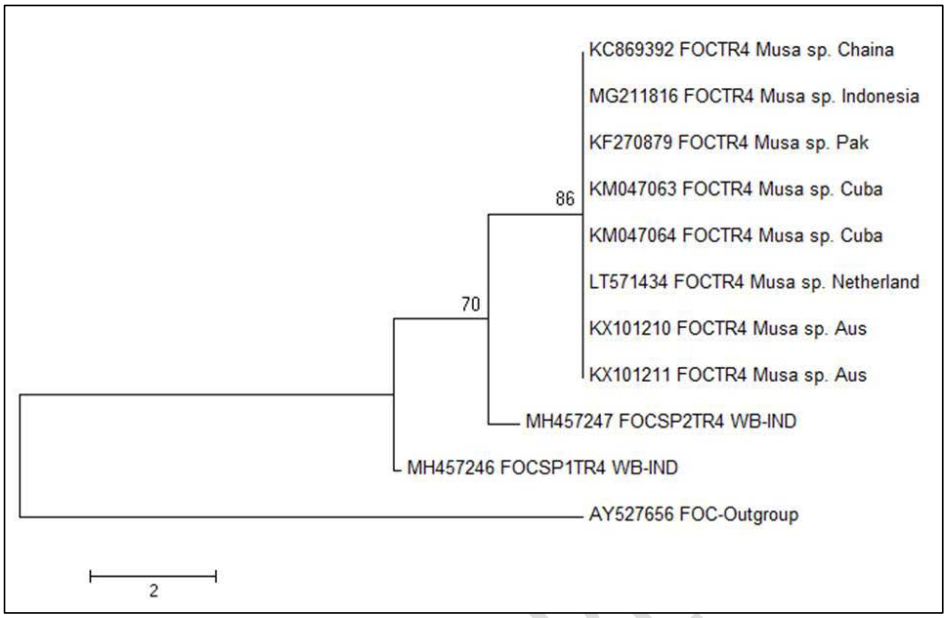

Fig. 8 Molecular Phylogenetic analysis using nucleotide sequences amplified using specific primer, the evolutionary history was inferred using the Neighbor-Joining method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Evolutionary analyses were conducted in MEGA7.