MOLECULAR CHARACTERIZATION OF Fusarium oxysporum F. sp. Cubense (FOC) TROPICAL RACE 4 CAUSING PANAMA DISEASE IN CAVENDISH BANANA IN PAKISTAN

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Fusarium wilt or Panama disease poses a great risk to banana production worldwide and is caused by Fusarium oxysporum f. sp. Cubense (Foc). Its tropical race 4 (TR4) is a major problem for Cavendish banana growing areas. Basrai (Dwarf Cavendish) is the major cultivated variety in Pakistan and now recently been reported to be affected by Foc TR4 in certain areas in the Sindh province. To genetically characterize, three independent isolates (P.TH1, P.TH2 and P.Th3) were sampled from different fields in district Thatta and fungus was isolated on potato dextrose agar. The pure fungal isolates were used to establish Koch’s postulates by testing their pathogenicity on tissue cultured Cavendish banana. A diagnostic PCR assay was optimized and used to sequence a reported molecular marker for specific detection of Foc TR4 and to sequence 28S-18S ribosomal RNA intergenic spacer region (IGS) from these local isolates. The phylogenetic of Pakistani isolates suggests that they belong to previously characterized lineage V of tropical race 4 of F. oxysporum cubense with 99% bootstrap support and show close relationship with Indonesian and Malaysian Foc TR4 isolates. The rapid spread of this disease in different banana growing areas of the Sindh is alarming and necessitates effective control of this through chemical and biocontrol, if remains unchecked, Foc TR4 can raise havoc to banana industry in Pakistan and would be devastating economically.

**Keywords:** Panama disease, Fusarium oxysporum f. sp. Cubense, tropical race 4, banana wilt, phylogenetic analysis.

INTRODUCTION

Banana is an important crop in agriculture economy of Pakistan. Major production (about 90%) comes from the Sindh province, while around 10% is produced in the Baluchistan. It is an export commodity; Pakistan earned 2.5392 million U.S. dollars by exporting 58,786 metric ton out of 96,545 metric ton total production from 22,098 hectares, in 2011 (http://faostat.fao.org). Banana was first introduced in the Sindh in 1913 from Bombay (India). Later many varieties were brought at different times from India, Sri Lanka and Bangladesh. Among them Dwarf Cavendish (Basrai) proved superior in Sindh climate and was released for general cropping in late 1950s (Khalid and Soomro, 1993; Bhatti, 1991). Currently this is the major cultivated variety, while William hybrid which was introduced first in 1969, then in 1978 and in 1985 from Queensland, Australia (Khalid and Soomro, 1993) and some Chinese varieties which are recently being introduced, cover less than 10% area under cultivation.

There are many economically important diseases of banana. These include bacterial diseases like bacterial soft rots of rhizome, pseudostem and bacterial wilt, while Banana bunchy top disease (BBTD) is the most devastating viral disease which is threatening Pakistan’s banana industry since 1991 (Hyder, 2009). Fungal specie i.e. Fusarium oxysporum f. sp. Cubense (Foc) causes fusarium wilt or Panama disease, which is highly damaging disease of banana (Bentley et al., 2001). Recently in 2012 a disease has been noticed in few banana fields of district Thatta and Hyderabad in the Sindh, affecting Dwarf Cavendish (Basrai) plantation (Syed et al., 2015), showing symptoms similar to Panama disease. Based on the morphology of isolated fungus from infected plants, Syed and colleagues (2015) identified the pathogen as Fusarium oxysporum f. sp. Cubense. In a parallel pioneering work, Ordonez and coworkers (2015) confirmed based on PCR diagnostics and mating experiments, that the tropical race 4 of Foc is causing Panama disease in Dwarf Cavendish plants in Pakistan (Ordonez et al., 2015).

Fusarium oxysporum Schlecht emend. Snyder & Hansen is a soil born, diverse and quite adaptive ascomycete fungus that includes pathogenic strains infecting human, animal and plants and a diverse range of nonpathogenic strains (Gordon and Martyn, 1997; Fourie et al., 2011). The plant pathogenic strains are divided into special forms or formae speciales...
based on host specificity (Fourie et al. 2011). The formae speciales Cubense of F. oxysporum infects banana and Heliconia spp. It is divided into various (more than 20) vegetative compatibility groups (VCGs) and four physiological races that infect different cultivars of banana. Race 1 infects ‘Gros Michel’ banana, while race 2 infects ‘Bluggoe’, race 3 infects Heliconia spp. and race 4 infects Cavendish banana and all banana cultivars susceptible to race 1 and 2 (Bentley et al., 1998). The fusarium wilt or Panama disease caused by Foc is one of the most devastating fungal diseases of banana. It was first reported in Australia in 1876 and believed to have been originated in Southeast Asia (Ploetz and Pegg, 1997), till 1950 it spread to all major banana producing regions of the world except few areas in the South Pacific, Mediterranean, Melanesia and Somalia (Ploetz, 2000). At that time, the major trade cultivar was Gros Michel which being susceptible to this disease, faced major epidemics. For example in the Ulua Valley of Honduras about 30,000 hectares were damaged during 1940 to 1960, area of about 4,000 hectares destroyed in Suriname in eight years and plantation in 6,000 hectare was lost during twelve years in Quepos area in Costa Rica (Ploetz, 2000). Due to similar destruction caused by Foc, the susceptible Gros Michel was replaced by Cavendish cultivars which were resistant to Foc except its race 4 which was thought to be a subtropical problem where low temperature predisposes the normally resistant Cavendish banana. The Foc of race 4 requiring low temperature and edaphic factors to cause disease in subtropics was called subtropical race 4 (STR4). However in 1992 a new variant of Foc known as tropical race 4 (TR4) was found infecting previously resistant Cavendish cultivars in tropical Southeast Asia and Australia (Ploetz, 2006). Recently Foc TR4 has been reported from Jorden (García-Bastidas et al., 2013), Lebanon and Pakistan (Ordonez et al., 2015; Syed et al., 2015). Dwarf Cavendish (Basrai) being the majorly grown cultivar and its susceptibility to Foc T4 necessitate the detailed genetic characterization of Foc TR4, which potentially can wreak a havoc in banana fields in Pakistan.

**MATERIALS AND METHODS**

**Sample collection and fungal growth:** Banana pseudostem of six to seven months old infected and healthy plants were sampled randomly, three plants each from three different fields in Thatta during 2014. Pseudostem were cut into 1-2 cm long pieces, surface sterilized with 5% sodium hypochlorite for 2 minutes and incubated on potato dextrose (PDA) agar with 50 µg/mL streptomycin at 25°C. After 48 hours mycelium growth was obtained from all infected plants, which then sub-cultured on PDA to obtain pure cultures.

**Establishment of Koch’s postulates:** The tissue culture raised Dwarf Cavendish (Basrai) banana having 4 to 6 leaves on average were planted in pots containing sterilized mixture of silt and baggase (1:1 ratio). One pure culture of fungus from each field was used to inoculate these banana plantlets for the confirmation of Koch’s postulates. The test plants were inoculated with Fusarium oxysporum f. sp. Cubense by mixing the pure culture from PDA agar to the medium in pots. Watering was done using sterile distilled water in alternate days and plants were kept in green house at temperature of 25-30°C. The symptoms were noticed 14 days post inoculation. The fungal isolates were again obtained on PDA agar as described earlier from artificially inoculated plants.

**DNA Isolation and PCR detection:** The DNA was extracted from field infected plants, their fungal isolates, artificially infected banana plants and their respective pure fungal cultures. For plant tissue, 400-500 mg tissue was processed while for fungal culture, a significant portion of a single colony, freed from traces of agar, was removed from the agar plat and transferred to a sterile Eppendorf tube with the help of a sterile scalpel and used for DNA isolation. Total DNA was extracted using modified CTAB method (Hyder et al., 2007), and then PCR amplified with Foc 1/2 (F: 5'-CAGGGGATGTATGAGGAGCT-3’ and R: 5'-'GTGACACGCGTGCTAGTTCC-3’) primer set which amplifies a fragment of a molecular marker (Lin et al., 2009) previously reported for specific detection of tropical race 4 of F. oxysporum cubense. FocTR4 F/R (F: 5'-CACGTTTAACGAGACTGAG-3’ and R: 5'-'CGCACGCCAGACTGCGTGGA-3’) primer set amplifies 28S-18S ribosomal RNA intergenic spacer region (Dita et al., 2010). EF2 F/R (F: 5'-ATGGGTAAGGARGACAAGC-3’ and R: 5'-'GGARGTACGAGTSATCATGTT-3’) primer set which amplifies Translation Elongation Factor 1α gene (Geiser et al., 2004). The β-actin primer set (F: 5'-ACTGTTCCCTATATACGAAAG-3’ and R: 5'-GAAGTGCTGACGAAGG-3’) and CP-16s rRNA F/R (F: 5'-ACGGGTAGTAAACCAGTAAG-3’ and R: 5'-CTTCCAGTACGGAACCTTG-3’) primer sets target banana nuclear actin and chloroplast 16s rRNA genes respectively as controls.

A typical PCR reaction contained about 50 ng DNA template, Taq buffer (10 mM Tris- HCl, pH 8.8, 50 mM KCl and 0.08 % Nonidet P40) 1.5 mM MgCl2, 200 µM of each dNTPs, 1.5 units Taq DNA Polymerase (recombinant) (Fermentas-Thermo Scientific USA), and 50 pM of each primer. The PCR thermal profile for all the reactions were pre-PCR denaturation at 96°C for 3 minutes followed by 35 cycles of denaturing at 96°C for 20 seconds, annealing at 52°C for 20 seconds and extension at 72°C for 40 seconds, and a final extension at 72°C for 20 minutes. The PCR products were analyzed using standard 1% agarose gel electrophoresis.
Molecular characterization of Foc TR4

**Sequencing and phylogenetic analysis:** The PCR products obtained through Foc 1/2 and FocTR4 F/R primers sets from one representative isolate of each field (designated as P.TH1, P.TH2 and P.TH3) were sequenced using commercial sequencing facility of Macrogen (Korea) using respective primers. The sequence data from both the strands of DNA was compiled using DNA Dragon Sequence Assembler version 1.5.1 (Sequentix-Digital DNA Processing, Germany) and used in phylogenetic analysis. Phylogenetic analysis was performed by aligning sequences of 28S-18S ribosomal RNA intergenic spacer region through MAFFT version 6.864 (Katoh *et al.*, 2002) and then obtaining Maximum Likelihood (ML) trees using Molecular Evolutionary Genetics Analysis Program (MEGA) version 5.05 (Tamura *et al.*, 2011). Phylogeny reconstruction test was performed using 1000 bootstrap replicates.

**RESULTS**

The plants sampled showed distinct symptoms of yellowing, splitting of leaves and severe wilting and cross sections of their pseudostem revealed reddish brown to dark brown coloration and rotting which progress from outer to inner leaf sheathe (Fig. 1). One representative isolate of each field (designated as P.TH1, P.TH2 and P.TH3) were used to obtain pure fungal cultures. When the sections of these infected and healthy pseudostem were surface sterilized and incubated on PDA agar mycelium growth was obtained from all infected plants but not from healthy plants. Pure fungal cultures generated by sub-culturing on PDA agar were used in inoculation trials on tissue culture raised Cavendish (Basrai) plants. The fungal inoculated plants showed symptom of yellowing and wilting at 14 days post inoculation while healthy controls which were given sterile water treatment did not show any symptom (Fig. 2). Pure fungal cultures were again isolated from these artificially inoculated plants and tested with PCR along with original field plants and their pure fungal isolations.

The PCR assay using Foc primers produced band of about 240 nt, FocTR4 produced specific band of about 460 nt and EF2 primers produced band of about 700 nt only from infected banana plant while bands of about 574 nt with β-actin gene specific primers and of about 1500 nt with chloroplast 16sRNA gene were produced from both infected plants. The total DNA extracted from infected and healthy banana plants were amplified using Foc and FocTR4 (both specific for *Fusarium oxysporum* f. sp. cubense tropical race 4) and elongation factor 2 (specific for *fusarium spp.*) primer sets. In addition, β-actin gene from banana nuclear genome and chloroplast 16s rRNA gene from banana chloroplast was also amplified using specific primers as positive controls. Foc primers produced band of about 240nt, FocTR4 produced specific band of about 460nt and EF2 primers produced band of about 700nt only from infected banana plant while bands of about 574nt with β-actin gene specific primers and of about 1500 with chloroplast 16sRNA gene were produced from both infected and healthy plants. A typical PCR reaction contained about 50 ng DNA template, Taq buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.08 % Nonident P40) 1.5 mM MgCl2, 200 μM of each dNTPs, 1.5 units Taq DNA Polymerase (recombinant) (Fermentas-Thermo Scientific USA), and 50 pM of each primer. The PCR thermal profile for all the reactions were pre-PCR denaturation at 96°C for 3 minutes followed by 35 cycles of denaturing at 96°C for 20 seconds, annealing at 52°C for 20 seconds and extension at 72°C for 40 seconds, and a final extension at 72°C for 20 minutes. M is O’RangeRuler™ 1 kb DNA Ladder (Fermentas-Thermo Scientific USA).
and healthy plants (Fig. 3). PCR produced the same results with the DNA isolated from pure fungal cultures and DNA from artificially inoculated plants. The sequence analysis of DNA fragment generated by Foc 1/2 primer set was identical in all the field isolates and their respective pure fungal cultures. The 209 bp sequence data submitted in GenBank (Accession No. KF270878) for P.TH1 showed 100% nucleotide identity through BLAS analysis (Altschul et al., 1997) with the sequence of a specific molecular marker (Lin et al., 2009) reported for specific detection of tropical race 4 of *F. oxysporum* cubense. Similarly, the 444 bp partial sequence of 28S-18S ribosomal RNA intergenic spacer region (Fig. 4).

Figure 4. Maximum Likelihood (ML) phylogenetic tree of *Fusarium oxysporum* f. sp. cubense based on partial 28S-18S ribosomal RNA intergenic spacer region. About 444 nt long partial 28S-18S ribosomal RNA intergenic spacer region, corresponding to the sequence of *Fusarium oxysporum* f. sp. cubense isolates P.TH1, P.TH2 and P.TH3 (sequenced in this study and highlighted in gray) were used to infer phylogenetic relations of different *Fusarium oxysporum* f. sp. cubense isolates using maximum likelihood method. All the Pakistani isolates showed close phylogenetic relationships (with 99% bootstrap support) with the Indonesian and Malaysian isolates belonging to Tropical race 4 in lineage-V with mating type locus (MAT) 1. Other isolates used to infer phylogeny are those which were used by Fourie and colleagues (2009) to define phylogenetic lineages in *Fusarium oxysporum* f. sp. cubense isolates. NRRL 28685 was used as out-group to root ML tree. Bootstrap values are shown on the branches. Bar indicates substitutions per site.
ribosomal RNA intergenic spacer, amplified with FocTR4 F/R primers, from three representative isolates i.e. P.TH1 P.TH2 and P.TH3 (Genbank accession numbers: KF270879, KP893342 and KP893343, respectively) was 100% identical in all the isolates and its phylogenetic analysis indicated that the Pakistani isolates belonged to previously (Fourie et al., 2009) characterized lineage V of tropical race 4 of *F. oxysporum* cubense with 99% bootstrap support (Fig. 4).

**DISCUSSION**

The Panama disease has done significant devastation of banana plantation around the world (Ploetz and Pegg, 1997; Ploetz, 2000). The presence of its tropical race 4 in Pakistan, which is a quarantine pathogen has very high economic importance and may adversely impact the trade earning of this crop. The accurate detection of this pathogen thorough some reliable method is very much important. Traditionally the Foc races were diagnosed using pathogenicity test and vegetative compatibility group (VCG) testing, however a major limitation was the unavailability of universally acceptable greenhouse inoculation procedure to characterize the Foc isolates (Bentley et al., 1998; Groenewald et al., 2006; Smith et al., 2008). Later on more specific diagnostic assay such as PCR based assay were developed (Dita et al., 2010) and use of molecular data such as sequence of intergenic spacer region (IGS) and translation elongation factor-1 alpha (TEF-1α) were standardized to study genetic diversity (O’Donnell et al., 2009; Fourie et al., 2009) and establishment of isolate identity (Bogale et al., 2007; Mehl and Epstein, 2007).

In Pakistan, the Foc has been diagnosed based on fungal morphology (Syed et al., 2015), PCR and VCG testing (Ordonez et al., 2015) but its genetic characterization based on phylogenetic analysis was never reported. In current research, we optimized a PCR assay, which utilized previously reported Foc 1/2 (Lin et al., 2009), FocTR4 F/R (Dita et al., 2010) and EF2 F/R (Geiser et al., 2004) primer sets along with newly developed {Dita, 2010 #645} {Dita, 2010 #645}β-actin F/R and CP-16s rRNA F/R primer sets for control amplification. We used this assay to generate sequence data for IGS region from three local Foc isolates which were tested for their pathogenicity on Cavendish banana by fulfilling Koch’s postulates and determined their phylogenetic relationships.

The Maximum Likelihood tree (Fig. 4) split into two clades, one comprises of lineages I to V, while other of lineages VI to VIII, previously identified by Fourie and colleagues (Fourie et al., 2009). All Pakistani isolates belonged to lineage V with mating type 1 and showed very close relationships with Tropical race 4 isolates of Foc originated from Indonesia and Malaysia. Other isolates in this clade belonging to rest of the lineages, are from Indonesia, Philippines, Australia, Honduras, USA, Brazil, Costa Rica, South Africa, Canary Island and Vietnam and are either of race 1, 4 of Subtropical race 4. The isolates in the second clade belongs to Australia, Philippines, Taiwan, Indonesia, Thailand, Malawi and Tanzania and either of race 1, 2 or Tropical race 4. Interestingly, the Tropical race 4 isolates in the lineage V split in the two clades; mating type 1 in first clade belonging to Indonesia, Malaysia and Pakistan. On the other hand, mating type 2 which originated from Taiwan, grouped in the second clade. This information could be useful in determining the possible origin of Foc T4 in Pakistan.

The history of Foc susceptible Cavendish banana cultivation in Pakistan, starts from the introduction of Basra (Dwarf Cavendish) variety from Bombay (India) by the Sindh Horticulture Research Institute (Bhatti 1991). This variety now has become the major cultivated variety in the country. Since its release for general cropping in Sind in the late 1950s, the productivity of this variety remained very high until a major epidemic of BBTV stuck in 1991 (Hyder, 2009; Hyder et al., 2007). Since then, the major pathogen of banana in Pakistan remained the BBTV. The import of banana from other countries is a practice of private farmers in Sindh, many of such imports never been reported but few, for which reports are available of transfer of suckers from Australia, have been implicated in their role of probable origin of BBTV in Pakistan (Hyder, 2009; Hyder et al., 2007; Hyder et al., 2011; Khalid and Soomro, 1993; Bhatti, 1991). The information available for BBTV could provide valuable insights for inferring the probable origin of Foc TR4 in Pakistan. The Foc TR4 has been reported in Taiwan (Buddenhagen, 2009; Molina et al., 2013; Sun, Su and Ko, 1978) and Malaysia in 1990s (Pin, 1996). In Indonesia its presence is established in many banana growing areas of the country including Java, Sumatra, Sulawesi, Halmahera, Kalimantan on the island of Borneo, and Papua Province (Davis et al., 2000; Hermanto et al., 2011). In mainland China it has wide distribution in Guangdong, Hainan, Guangxi, Fujian and Yunnan provinces (Qi, 2001; Qi et al., 2008). It has been reported in the island of Mindanao in Philippines (Molina et al., 2008). In India, though Foc TR4 has not yet been reported but considerable diversity has been found in the population Foc in the country (Thangavelu and Mustaffa, 2010; Mustaffa and Thangavelu, 2011; Thangavelu et al., 2012). It has also been found in Jordan (García-Bastidas et al., 2013), in Mozambique (Butler, 2013), Lebanon (Ordonez et al., 2015) and in Australia (Condé and Pitkethley, 2001; Cronin, 2015). Presence of Foc TR4 in Australia and reports of import of sucker from there to Pakistan by private farmers might provide a clue about the probable origin of TR4 in Pakistan but the close association of Pakistani Foc TR4 isolates with Indonesian and Malaysian isolates weaken this assumption. Nevertheless until the clue of transfer of planting material from Indonesia and Malaysia is not obtained, origin of Pakistani problem of Foc TR4 cannot be accretion.
In Pakistan, the first observation of yellowing and wilting disease in Sindh was in 2012 in Baoo Pooran area (Ordonez et al., 2015) of District Thatta. This area had been affected by flooding during 2008 and 2010 which might have helped the establishment of this disease in the area. And now the report of observation of this disease in district Hyderabad (Syed et al., 2015), suggests a rapid spread to other banana growing areas in the Sindh. Banana is vegetatively propagated and transfer of its sucker among the fields is very common practice in Pakistan. The situation is quite alarming and requires a quick response to address the problem on national level. Dedicated efforts to perfume a diagnostic survey, elimination of infected plants and use of chemical and biocontrol to eliminate the disease on immediate basis is a necessity of time. Otherwise if this disease is continue to spread to other areas, it can cause a serious havoc to banana production in Pakistan with serious consequences for local market, export and social well-being of farmers, who still largely depend on banana for their earnings.

**Conclusion:** Panama disease is one of the most devastating fungal disease of banana and its tropical race 4, causes this disease to Cavendish banana, which is predominant variety cultivated in Pakistan. The recent reports of the presence of Foc TR4 in Pakistan, necessitate characterization of this pathogen on genetic level. To this end, the Foc TR4 was detected through a PCR based assay, Koch’s postulates were established and sequencing and phylogenetic analysis of three isolates were performed which suggested its close association with Indonesian and Malaysian isolates. The rapid spreading of this disease in different banana growing areas in the Sindh, necessitates quick remedial actions, otherwise it can severely threat the banana production in Pakistan.

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