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ISOLATION AND IDENTIFICATION OF PATHOGENIC FUNGI FROM BANANA FIELDS

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ABSTRACT

Banana is one of the oldest fruit crop belonging to the genus Musa which supports livelihood of millions of people. Global banana production is severely affected by the fungal pathogens among which Panama disease is the major problem to banana industry. This study was carried out to survey Fusarium wilt in banana fields near Bakrol village. During 2014-15, two Fusarium sp. were isolated from two different fields and identified by molecular technique. The pathogenicity test on detached banana leaves and pots were carried out. Fusarium sp. isolated from fruits showed symptomatic yellow leaf spots and necrosis within ten days after spore inoculation while no symptoms were observed with pseudostem isolate and distilled water inoculated leaves. The information about the occurrence of disease may be useful for developing resistant banana cultivars and adopting the appropriate agronomic practices to control the disease.

Keywords: Banana, Fusarium oxysporum f.sp. cubense, Panama disease, Tropical race 4, Plant pathogenic fungi.

INTRODUCTION

Bananas (*Musa* spp.) are giant perennial herbs belonging to the order Zingiberales of the monocotyledon group and originated in Southeast Asia and the Western Pacific. The cultivated bananas have evolved from the hybridization of wild species of *M. acuminata* and *M. balbisiana* [1]. Banana provides more balanced food than any other fruits or vegetable for millions of people across the globe. Bananas serve as an ideal and low cost staple food source for developing countries [2]. India is the largest producer of banana in the world contributing 37.2% of total world production [3]. Banana occupies only 11.1% of total cultivated area under fruits while contributing 32.6% of total fruit production in India. Gujarat is the second largest producer of banana following Tamilnadu [4].

Global banana production is adversely affected by the re-emergence of a Fusarium wilt [5]. Fusarium wilt (also known as "Panama disease") is a devastating disease caused by fungi *Fusarium oxysporum* f. sp. *cubense* (Foc) [6]. It is a soil borne pathogen which infects the root system and goes on to colonize the plant through the vascular system, subsequently occludes the xylem vessels. The hyphae of the fungus can even reach the leaves leading to wilting and death of banana plant. However, one of the worst effects of Panama disease is the production of chlamydospores or resting spores which survive in the soil for decades and cannot be controlled by fungicides. As soon as a susceptible banana plant grows nearby, these spores germinate, infect the plant, and kill it. However, only two races namely Foc race 1 and race 4 have been shown to endanger banana production worldwide [7]. The tropical race 4 strain of Fusarium wilt is regarded as one of the most serious threats to banana production since disease resistant varieties, for replacing Cavendish which are currently the source of 99% of banana exports, are not yet widely available [8].

Therefore, this study was carried out for detection and identification of Fusarium wilt in diseased banana plants in the fields near Bakrol village (22°33'50"N, 72°54'40"E), Anand, Gujarat, India.

METHODOLOGY

Sample collection and isolation of pathogens

The survey was carried out for fungal diseases in the banana fields near Bakrol, Gujarat in the

year 2014-15. The samples of infected pseudostem and fruit bunch of Grand naine from different fields were collected and brought to the lab in the air tight polybags. The vascular tissue showing symptomatic reddish brown lesions from pseudostem was cut in to small pieces, surface sterilized and inoculated on PDA media. Similarly, pieces of infected fruit peel showing surface grown mycelia were collected and placed over PDA medium [9]. After incubation for 3-5 days at 27±2°C temperature, the pinkish white cottony colonies of *Fusarium* sp. were isolated and subcultured. Pure cultures of the isolates were stored at 4°C in refrigerator for further study.

Cultural studies

The actively growing cultures of both isolates were evaluated for morphological characteristics. Actively growing mycelia were suspended in sterile distilled water and agitated to release spores. Conidial morphology was observed by lactophenol cotton blue staining under (x40 and x100) light microscope and spore size was measured using calibrated micrometer.

Identification by molecular technique

The consensus sequence of D2 region of LSU gene was generated by amplification of 28S rDNA fragment using the primers (DF-ACCCGCTGAACTTAAGC and DR-GGTCCGTGTTTCAAGACGG) similar to LROR and NL4. Based on maximum identity score first fifteen sequences were aligned using Clustal W. The phylogenetic tree was constructed using MEGA5 [10]. Sequence of both fungi has been submitted to NCBI GeneBank.

Pathogenicity test using detached leaf assay

To confirm pathogenicity, detached banana leaf assay was carried out. Spore suspensions were prepared by suspending mycelial mat into sterile distilled water followed by agitation. Spore counting was carried out by placing a drop of spore suspension on Neubauer chamber and spore concentration was adjusted to working concentration of 10^4 spores/ml.

The fresh and healthy leaves of three to four months old banana plants were surface sterilized with 70% alcohol for 1 minute in aseptic condition and washed with sterile distilled water for several times. Leaves rubbed with sterile blades to remove waxy layer from the surface were inoculated with spore suspension (10^4 spores/ml) of each fungus separately in sterile petriplates containing wet Whatman filter paper. These plates were incubated under dark-light condition at room temperature. The plates were daily observed for characteristic disease symptoms on detached banana leaves as observed on original plants in the fields. The experiment was conducted in triplicate.

RESULTS AND DISCUSSION

In the study, *Fusarium* sp. from two different fields of Bakrol village were isolated during the year 2014-15. In September 2014, the heavily infected banana pseudostem with yellowing and wilting of leaves which caused death of whole plant within two months after planting (Figure 1 A) was collected from field 1. *Fusarium* infected bunch with yellow necrosis of banana fingers was observed on healthy looking plant and collected in June 2015 from field 2 (Figure 1 B).

On the basis of colony characteristics and microscopic study, the isolates from both samples were found to be *Fusarium* sp. The pinkish white cottony colonies showing reddish pigmentation underside were isolated as shown in figure 2 A and C. The growth of *Fusarium* sp. FoF isolated from fruits was more and appeared fluffy as compared to that of *Fusarium* sp. FoS isolated from pseudostem.

The spore showed the characteristic sickle shaped, 3-4 septate macroconidia and oval shaped microconidia under the light microscope (figure 2 B and D) when stained using lactophenol cotton blue. The size of macroconidia from both isolates varied from 18-30 × 3-3.8 µm. Fusarium sp. causing banana fruit rot was isolated from local market of Anand with cottony, pinkish white mycelial growth on PDA medium [11]. Significant variations were observed in cultural characteristics, production of microconidia, macroconidia and chlamydospores and also colony pigmentation from white to pinkish in the

Fusarium isolates from various pseudostem bits of infected banana plants [9]. The different isolates of *Fusarium oxysporum* f. sp. *ciceri* causing wilting of chickpea isolated from various region of West Bengal also showed differences in the growth of colony, color of pigment produced and size of macro- and micro-conidia [12]. A diverse population of *F. oxysporum* isolated from different tomato fields of Uttar Pradesh showed the morphological and cultural differences as well as large variability appeared in conidial morphology [13]. *Fusarium* sp. isolated from fruits were showed fluffier mycelial growth.

The sequences of 852 bp and 621 bp of D2 region of 28S rDNA (LSU) gene were generated from forward and reverse sequences obtained by amplification of 28S rDNA fragment from both the fungi isolated from field 1 and 2 respectively. The phylogenetic tree constructed showed the maximum identity (100%) of these fungi with *Fusarium* sp. KF215 (Accession number: KM096153.1) and *Fusarium* sp. NRRL45996 (Accession number: GQ505760.1) respectively for the isolates *Fusarium* sp. FoS and FoF (figure 3- A and B). The nucleotide sequences of both isolates were submitted to the NCBI GenBank and the accession numbers allotted are KY548401 and KY548402 for the *Fusarium* isolated from pseudostem FoS (field 1) and fruits FoF (field 2) respectively. Foc race identification is cumbersome therefore other methods unveiling genetic diversity can be used. Species specific primer sets FOF1 and FOR1 have been used to detect *F. oxysporum* strains in tomato [13]. Dita et al. [14] developed a PCR based diagnostic tool specifically to detect Foc Tropical race 4 which is presently the best option for the rapid and reliable detection of TR4. Using this PCR based molecular tool, Ordonez et al., [15] has reported the TR4 causing Panama disease in Cavendish bananas in Pakistan and Lebanon in 2016. In India, the dominant VCG 0124/5 complex was isolated from ABB bananas i.e. Monthan and the Pisang Awak cultivar Karpooravalli, AB bananas i.e. Ney Poovan and the Silk cultivars Rasthali, Malbhog and Mortaman (AAB). The cultivars from which most VCGs were collected in India were Karpooravalli and Ney Poovan and not a single isolate from Cavendish bananas [16]. TR4 has been reported to infect Cavendish variety in Jordan, Lebanon, Oman, Pakistan [17] and recently in Australia [18]. The *Fusarium* isolates in this study do not match the characteristics of Tropical Race 4.

In detached banana leaf assay as shown in Figure 4, minor 1-2 cm size blackish lesion was observed within ten days after spore inoculation of *Fusarium* sp. FoF (KY548402) in wounded leaves, showing similar symptoms as observed on infected fruits. While no symptoms were observed with *Fusarium* sp. FoS (KY548401) strain isolated from pseudostem and distilled water inoculated leaves. The same fungus was consistently reisolated from the symptomatic leaf tissue. Thus, detached leaf assay revealed that *Fusarium* sp. isolated from fruits are more virulent than that isolated from pseudostem. The detached leaf assay in petri dishes which is the easiest and most reliable laboratory test was used to evaluate virulence and pathogenicity of numerous strains of *Pseudomonas syringae* pv. *syringae* on Pear [19]. The effectiveness of the detached leaf assay was found to be similar with whole seedling assay and applied for assessing wheat genotypes against leaf rust [20]. Detached leaf assay, a rapid and inexpensive method, demonstrated a relationship between pathogenic *Pythium* inoculum concentration in soil and the expression of root rot symptoms in *Chrysanthemum* [21]. Wounded and unwounded *in vitro* detached leaf assay demonstrated that *Fusarium langsethiae* is a pathogen of wheat and oats and may have developed some host preference towards oats [22]. In this study the *Fusarium* isolates FoF (KY548402) appeared to be more virulent than *Fusarium* sp. FoS (KY548401) isolate.

CONCLUSION

Detection of Fusarium wilt in various region is the threatening signal for Cavendish production which is a major component of local agriculture economies in India and the world. The losses will increase without a heightened awareness of the threat that TR4 poses and the execution of actions to prevent its spread. A systematic understanding of Foc epidemiology and pathology is immediately required so as to

develop effective methods to destroy infected plants and (biological) soil treatments.

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Figure 1: Diseased banana plants (A) banana pseudostem showing typical symptoms of wilt and necrosis. (B) Banana bunch showing yellowing and necrosis of fruits.

Figure 2: (A, C) Isolated pinkish white cottony colonies of *Fusarium* sp. and (B, D) photomicrograph showing sickle shaped spores of *Fusarium* sp.

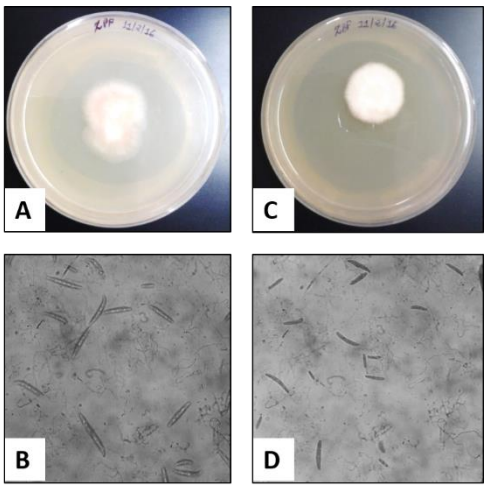

Figure 3: Phylogenetic analysis of isolates KY548401 and KY548402 showing maximum similarity of BLAST analysis.

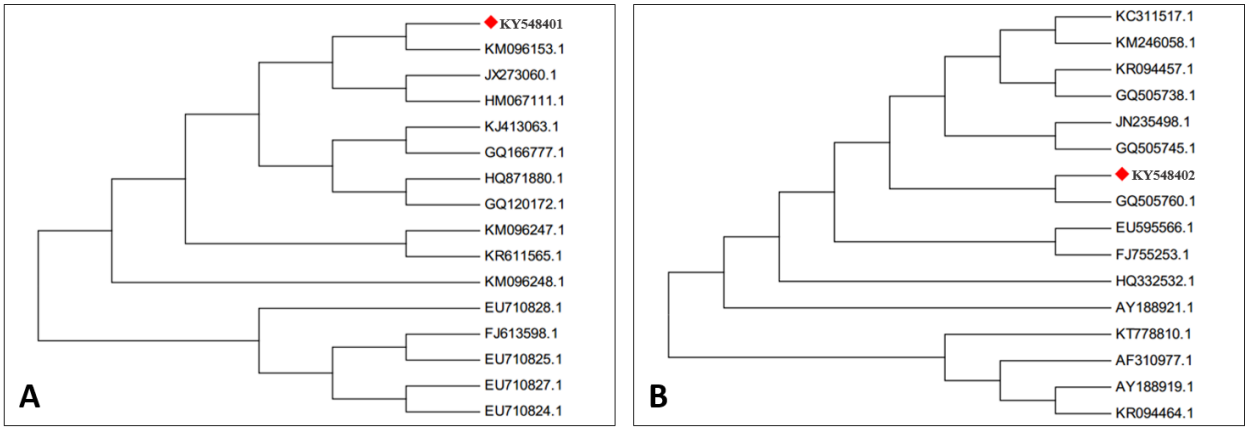

Figure 4: Pathogenicity testing on detached banana leaf pieces indicating symptomatic necrosis upon spore inoculation of isolated *Fusarium* sp.