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CHARACTERIZATION OF *COLLETOTRICHUM FALCATUM* WENT. CAUSING RED ROT IN SUGARCANE *SACCHARUM* COMPLEX

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ABSTRACT
Red rot of sugarcane caused by *Colletotrichum falcatum* Went. is a serious problem which damaged the most of the important varieties. Various isolates of *C. falcatum* produced cottony, fluffy, fluffy cottony or suppressed growth on the PDA with colour range of grey, greyish black to greyish white. The isolate SGCF-8 showed maximum growth (87.34 mm) on PDA when incubated at 27 ± 2ºC temperature for 10 days. The isolate SGCF-5 was showed maximum sporulation (24.67 million spores/ml) while maximum dry mycelial weight was observed in isolate SGCF-1 (184.67mg) on PDB incubated at 27 ± 2ºC temperature for 15 days. The molecular characterization study was carried out by using five ISSR primers. The primer UBC-873 (92.30%) was showed the highest percentage of polymorphism with polymorphic information content (PIC) of 0.6853846. The dendrogram was showed isolate (SGCF-4) was situated separately with similarity matrix of 0.407 with SGCF-1. Other nine isolates were clustered in one group and sub-groups.

INTRODUCTION

Colletotrichum falcatum Went. is a very important pathogen responsible for major economic losses caused by red rot in sugarcane worldwide. It belongs to Glomerallaceae of Asocmycota. Its telomorph is *Glomeralla tucumanesis* (Rafay and Singh, 1957). Red rot of sugarcane is distributed worldwide, but is found mainly in subtropical and tropical regions. Red rot disease caused by *C. falcatum* is one of the most serious threats to sugarcane cultivation in India (Beniwal *et al*., 1989 and Satyavir, 2003). So far diseases of sugarcane reported in India, among them red rot has caused much damage in sub-tropical areas including complete crop loss. These losses are caused by the degradation of the cuttings and stools, death of the stalks and stools and reduction in sugar content and purity. No genotypes could stand erect against the threat of red rot for a long time. Probably this is the reason that entire sugarcane breeding in Asia is geared up around the red rot disease. Resistance sugarcane varieties to red rot released for general cultivation may become susceptible after 5-6 years. The breakdown of red rot resistance may be due to evolving of new virulent gene in *C. falcatum*. This phenotypic identification is time consuming, expertise specific and not always fully discriminative. In the recent past, various DNA-based characterization in many Colletotrichum species have been tested by using intergenic spacer region of the ribosomal DNA repeats in *C. graminicola* (Latha *et al*., 2003), random amplified polymorphic DNA (RAPD) in *C. lindemuthianum* in common bean (Padder *et al.,* 2007), RAPD and restriction fragment length polymorphism (RFLP) of non-long terminal repeats retrotransposon in *C. gloesporioides* (Kelemu *et al.,* 1999), mating compatibility, intronic and mitochondrial DNA sequences in *C. acutatum* by Guerber *et al.*, 2003. Recently, *Colletotrichum* has been unveiled as a polyphyletic taxon, with strains exhibiting considerable sequence diversity and spanning *C. caudatum* and *C. sublineolum* as phylogenetic species (Crouch *et al.,* 2008). It is therefore very important to continue monitoring of diseases and the study of genetic variability of pathogens should contribute to the understanding of pathogen evolution. In sugarcane plant, considering the breakdown of resistance to red rot, the present investigation on characterization in *C. falcatum* has been undertaken. The objectives behind this work were (i) to understand pathogenicity behaviour (ii) characterization of genetic variability in *C. falcatum* isolates of sugarcane using inter simple sequence repeat (ISSR).

MATERIALS AND METHODS

Fungal isolation and maintenance

Ten different isolates of *C. falcatum* were isolated from ten different varieties from different location from Gujarat state sugarcane growing area. These cultures were maintained on PDA slant at 4ºC temperature for further study.

Pathogenic variability

The pathogenic variability study was conducted at the field of Regional Sugarcane Research Station. According to AICRP programs, the pathogenic variation study were studied with fourteen differential varieties (SES 594, Co 419, Co 975, Co 997, CoS 767, Baragua, Co 7717, Co 8436, Khakai, Co 62399, Co 1148, Co 6013, Bo 91, CoJ 65). The different isolates

collected from different locations were grown on PDA by single mycelial tip method for ten days at 27 ± 2°C temperature. After ten days of growth, spore suspension of 1x10^6 spores/mL was prepared for inoculation. The inoculation was done on seven month old sugarcane with plug method of inoculation. Disease reactions were scored on the basis of differential disease index as

Resistant (0-2), Intermediate (2.1-5.0) and Susceptible (5.1-9.0) groups of the host (Bailey and Jeger 1992).

Cultural and morphological variation

The isolates were separately cultured on Potato dextrose agar (PDA) media, at 27 ± 2°C for seven days and diameter of the mycelial growth and the growth pattern were recorded. The isolates were also cultured in liquid media in potato dextrose broth (PDB). After fifteen days of incubation the culture were filtered out with Whatman No. 1 filter paper and spores were counted with Haemocytometer.

By the micrometry method size of conidia, conidiophore, acervuli, setae and appresoria were measured.

Molecular variation

Study of molecular variation by using ISSR primers

Genomic DNA isolation and purification

Ten purified isolates of *C. falcatum* were grown on potato dextrose broth at 27 ± 2ºC for 10 days. Young growing mycelia were filtered through autoclaved muslin cloth, and about 1g mycelia were ground in a pre-chilled mortar with liquid nitrogen. The powder was transferred to an Eppendorf tube and resuspended in 500 µL of extraction buffer (0.5% SDS, 200 mM Tris-HCl pH 8.5, 250 mM NaCl, and 25 mM EDTA) (Raeder and Broda 1985). The tubes were incubated at 65°C for 30 min with occasional gentle swirling. Two third volumes of chloroform: isoamylalcohol (24:1, v/v) was added to this sample and mixed for 15-25 min and then centrifuged at 10000 rpm for 10 min. For purification, 10 µL RNAse (10 mg/mL) was added to the supernatant and incubated for 30 min at 37ºC. The incubated sample was again extracted with chloroform: isoamyl alcohol solution and centrifuged at 10000 rpm for 10 min. Chilled isopropanol (0.57 volume) was added to the upper phase, mixed by inverting and centrifuged at 10000 rpm for 10 min at 4°C. The DNA pellet was rinsed twice with 70% ethanol for 10-15 min. and then dried at room temperature. The dried pellet was dissolved in 250 µL TE buffer (pH 8.0). The purified DNA was checked by running 2 µL of the sample on a 0.8% agarose gel. The quality and quantity of DNA were measured at 260/280 nm and 260nm respectively using nanospectophotometere.

Molecular analysis

Twenty ISSR primers were screened for the amplification of genomic DNA with one isolate of *C. falcatum*, five of them gave reproducible results. The annealing temperature of primers varied according to GC content.

For ISSR, PCR amplifications were carried out in a reaction volume of 25 µL containing 25 ng of fungal DNA. The PCR reaction mixture consisted of 10X PCR buffer (0.01% gelatin 20 mm Tris–HCl, pH 8.4; 50 mM KCl, 1.5 mM $MgCl_2$), 10 mM dNTPs, 1 U Taq DNA polymerase, and 0.2 µM primer. All the

Table 1: Different isolates of *Colletotrichum falcatum* Went., causing red rot in sugarcane collected from different places and geographical locations

Sr. No.	Isolate	Variety	Place	Geographical location
1.	SGCF-1	Bo 138	Regional Sugarcane Research Station, Navsari Agricultural University, Navsari.	20°55′ 10.89" N 72°53′ 31.30"E
2.	SGCF-2	Co 86002	Baroda	22°12′ 48.26"N 73°11′ 06.27"E
3.	SGCF-3	Co 86002	Bardoli	21°06′ 50.63"N 73°05′ 46.07"E
4.	SGCF-4	Co 86032	Vyara	21°07′ 16.56"N 73°22′ 25.59"E
5.	SGCF-5	CoC 671	Gandevi	20°48′ 12.79"N 73°00′ 35.08"E
6.	SGCF-6	CoN 05071	Regional Sugarcane Research Station, Navsari Agricultural University, Navsari.	20°55′ 10.54"N 72°53′ 30.27"E
7.	SGCF-7	Co 8014	Bharuch	21°44′ 27.02"N 73°05′ 09.53"E
8.	SGCF-8	Co 7527	Vataria	21°45′ 00.91"N 73°11′ 28.54"E
9.	SGCF-9	Co 92004	Palsana	21°05′ 29.62"N 72°58′ 08.04"E
10.	SGCF-10	Co 97002	Valsad	20°37′ 13.74"N 72°57′ 25.06"E

Table 2: Details of amplification obtained with different ISSR primers

Sr. No.	Name of primer	Sequence	Annealing temperature ºC	No. of total bands	No. of polymorphic bands	No. of monomorphic bands	Polymorphism percent (P %)	Total No. of bands amplified in 10 isolates	PIC value
1.	UBC-821	5′-$(GT)_8$ T-3′	50.3	5	4	1	80.00	26	0.644
2.	UBC-825	5′-$(AC)_8$ T-3′	51.4	7	6	1	85.71	51	0.447
3.	UBC-850	5′-$(GT)_8$ YC-3′	52.7	8	7	1	87.50	50	0.54
4.	UBC-857	5′-$(AC)_8$ YG-3′	54.3	7	4	3	57.14	54	0.345
5.	UBC-873	5′-$(GACA)_4$-3′	43.9	13	12	1	92.30	73	0.685
Total				40	33	7	82.50	254	2.662

PCR reaction components were obtained from Bangalore Genei Pvt. Ltd. Bangalore, India. The amplifications were performed using a Thermal cycler (T-Gradient Biometra; GmBh, Goettingen, Germany). For ISSR markers, PCR temperature profiles were as follows: initial DNA denaturation at 94ºC for 5 min, followed by 35 PCR cycles at 94ºC for 1 min, 1 min at the primer specific annealing temperature 72ºC for 2 min and a final cycle at 72ºC for 7 min. All the amplified PCR products were resolved by electrophoresis in 1.4% Agarose gel containing 5μg ethidium bromide/100mL for 2 h in 0.5X TBE buffer at 60 V. the standard DNA marker (Fermentus) was also run along with the sample. The separate bands were visualized under UV light and photographed by Biorad gel documentation system (Biorad GelDoc XR+).

RESULTS

Pathological, Cultural and Morphological variation:

Pathological variation

Pathogenic variability of ten isolates were carried out on a set of 14 differential varieties by using plug method of inoculation as adopted under AICRP sugarcane. The data of the disease score indicated the differential reactions on host genotype. Isolates of *C. falcatum* indicating, thereby the differential pathogenic behavior within isolates.

The data of the disease score recorded during 2010-11 crop season indicated that all the ten isolates showed more or less pathogenic behaviour against fourteen differentials (Table 3). In the present study, all the isolates were showed moderately susceptible to highly susceptible reaction against variety Co 429, Co 975, Baragua and Khakai. Isolate SGCF-2, SGCF-9 and SGCF-10, SGCF-7, SGCF-2 and SGCF-7 showed resistance reaction against variety SES 594, Co 7717, Co 06013, Co 91and CoJ 64 respectively. All the ten isolates were showed intermediate reaction against rest of all differentials (Table 3).

Molecular variation

Identification and evaluation of ISSR markers for diversity estimates in ten *C. falcatum* isolates

In recent years, molecular markers have received arable attention and have been used for genetic diversity phylogenetic and evolutionary studies and mapping and tagging of agronomically important traits in different plant species. It can also be used in the study of molecular diversity in microorganism such as fungus, bacteria, viruses, etc. In this study, the present investigation was carried out with the ten different isolates of fungus *C. falcatum* to study the molecular variation in these isolates.

A total of 20 Primers consisted of di-nucleotide repeat motifs were used for initial screening with two isolates. Out of these, fifteen primers gave no amplification at all, while only five primers were found to give clear banding patterns and were subsequently used to analyze the entire set of ten isolates. These five ISSR primers amplified a total of 40 scorable bands of which 33 bands were polymorphic. These primers showed variation in the percentage of polymorphism. The percent of polymorphism ranged from 57.14% to 92.30%. The primer UBC-873 showed the highest value of percentage of polymorphism (92.30%). The polymorphic information content (PIC) value of these five primers ranged from 0.3457143 to 0.6853846.

Amplification pattern obtained using primers UBC-821, UBC-825, UBC-850, UBC-857 and UBC-873 are depicted as representative pictures in figure 2. This primer UBC-821 has amplified a total number of five bands. Out of these five bands in four were polymorphic while one was monomorphic amplification pattern. In same Plate, amplification pattern of the primer UBC-825 showed a total of seven scorable bands, out of which six were polymorphic. A total of eight bands were amplified on primer UBC-850 shows in, out of which seven were polymorphic while one was monomorphic. The banding pattern of primer UBC- 857 was showed total of seven scorable bands out of which four were polymorphic while three were monomorphic. The primer UBC-873 was showed more amplification than other primers with thirteen scorable bands of which twelve were polymorphic and one was monomorphic.

The ISSR amplification data were used to obtain a similarity

Table 3: Pathogenic variability of the isolates of *C. falcatum* against fourteen host differentials

Isolates	Bo 138 (Cf-1)	Co 86002 (Cf-2)	Co 86002 (Cf-3)	Co 86032 (Cf-4)	CoC 671 (Cf-5)	CoN 05071 (Cf-6)	Co 8014 (Cf-7)	Co 7527 (Cf-8)	Co 94012 (Cf-9)	Co 97002 (Cf-10)
Differentials										
SES 594	(3.0) I	(2.0) R	(4.5) I	(6.0) S	(3.5) I	(4.5) I	(4.5) I	(2.5) I	(3.0) I	(5.5) S
Co 419	(6.5) S	(6.5) S	(5.5) S	(6.0) S	(7.5) S	(4.5) I	(5.5) S	(7.5) S	(7.5) S	(6.0) S
Co 975	(8.5) S	(8.0) S	(9.0) S	(5.5) S	(6.5) S	(5.5) S	(7.0) S	(7.5) S	(8.0) S	(6.5) S
Co 997	(8.0) S	(9.0) S	(5.0) I	(9.0) S	(9.0) S	(7.5) S	(7.5) S	(3.3) I	(7.5) S	(4.5) I
Co 767	(4.0) I	(3.5) I	(8.5) S	(4.5) I	(7.5) S	(3.5) I	(5.5) S	(3.0) I	(3.0) I	(4.0) I
Baragua	(5.0) I	(6.0) S	(4.0) I	(5.0) I	(5.0) I	(4.0) I	(7.0) S	(8.0) S	(6.0) S	(5.0) I
Co 7717	(8.0) S	(6.0) S	(3.5) I	(6.5) S	(6.0) S	(3.0) I	(3.0) I	(2.5) I	(2.0) R	(2.0) R
Co 8436	(6.5) S	(7.5) S	(6.5) S	(4.0) I	(7.5) S	(3.0) I	(5.0) I	(6.0) S	(6.0) S	(4.0)I
Khakai	(4.5) I	(8.0) S	(8.0) S	(8.0) S	(6.5) S	(4.5) I	(7.5) S	(6.5) S	(8.0) S	(7.5) S
Co 62399	(5.0) I	(3.0) I	(3.5) I	(4.5) I	(3.5) I	(3.5) I	(3.0)I	(3.5) I	(3.0) I	(4.5) I
Co 1148	(6.0) S	(9.0) S	(8.5) S	(9.0) S	(6.0) S	(4.0) I	(7.5) S	(4.0) I	(8.5) S	(8.5) S
Co 06013	(6.0) S	(8.5) S	(8.5) S	(8.5) S	(8.0) S	(8.0) S	(2.0) R	(2.5) I	(8.5) S	(7.0) S
Bo 91	(2.5) I	(2.0) R	(3.0)I	(4.5) I	(4.5) I	(4.0) I	(2.5) I	(3.5) I	(2.5) I	(3.5)I
CoJ 64	(6.0) S	(7.0) S	(4.5) I	(6.0) S	(4.0)I	(5.5) S	(2.0) R	(3.0)I	(5.0) I	(5.0) I

R = Resistant; I = Intermediate & S = Susceptible

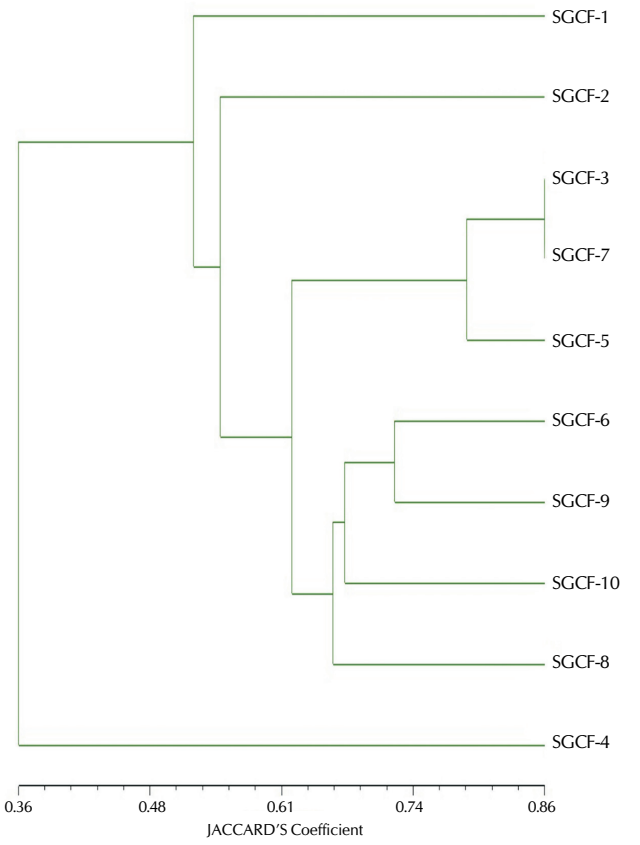

Figure 1: Dendrogram of average analysis of combined Inter Simple Sequence Repeat(ISSR) primers from 10 isolates of *C. falcatum* Went.

matrix. A dendrogram was constructed using UPGMA method for ten isolates under present study. The dendogram clearly indicated the one big cluster of nine isolates while one isolate (SGCF-4) was separately situated with similarity matrix of 0.407 with SGCF-1. In these nine isolates SGCF-1 and SGCF-2 were placed separately while others were sub divided in two sub clusters A and B. The cluster A was contain three isolates (SGCF-3, SGCF-5 and SGCF-7) while cluster B contain four isolates (SGCF-6, SGCF-8, SGCF-9 and SGCF-10). These two sub clusters showed similarity coefficient of 0.61. The sub-cluster A consisted of isolates SGCF-3 and SGCF-7 which were most similar which were diversed from isolate SGCF-5.

DISCUSSION

The phenomenon thus proved that on isolate pathogenic to one variety may not be identical in reaction with another variety. Thus result indicated that the response of isolates varied with genotype. The phenomenon endorses supports the observations of Khirabat, *et al*. (1980) and Singh, *et al*. (1984). The study conducted by Ram Ji Lal, *et al*. (1989) suggested that the isolates of red rot pathogen with differential pathogenicity were in existence in the country. It appears necessary to exercise much great care and discretion in selection of *C. falcatum* isolates for screening sugarcane varieties. It appears desirable to make regular collection of prevailing isolates of red rot pathogen in nature to keep the track for determining the existence and changes in population of parasitic races in the country or region.

Pathogenicity tests divided the pathogenic potential of *C. falcatum* into low, medium and high-virulence groups. It clearly revealed that ten isolates of *C. falcatum* inoculated on fourteen standard differentials were the most virulent isolates. Pathogenicity behaviour is supported by the earlier studies

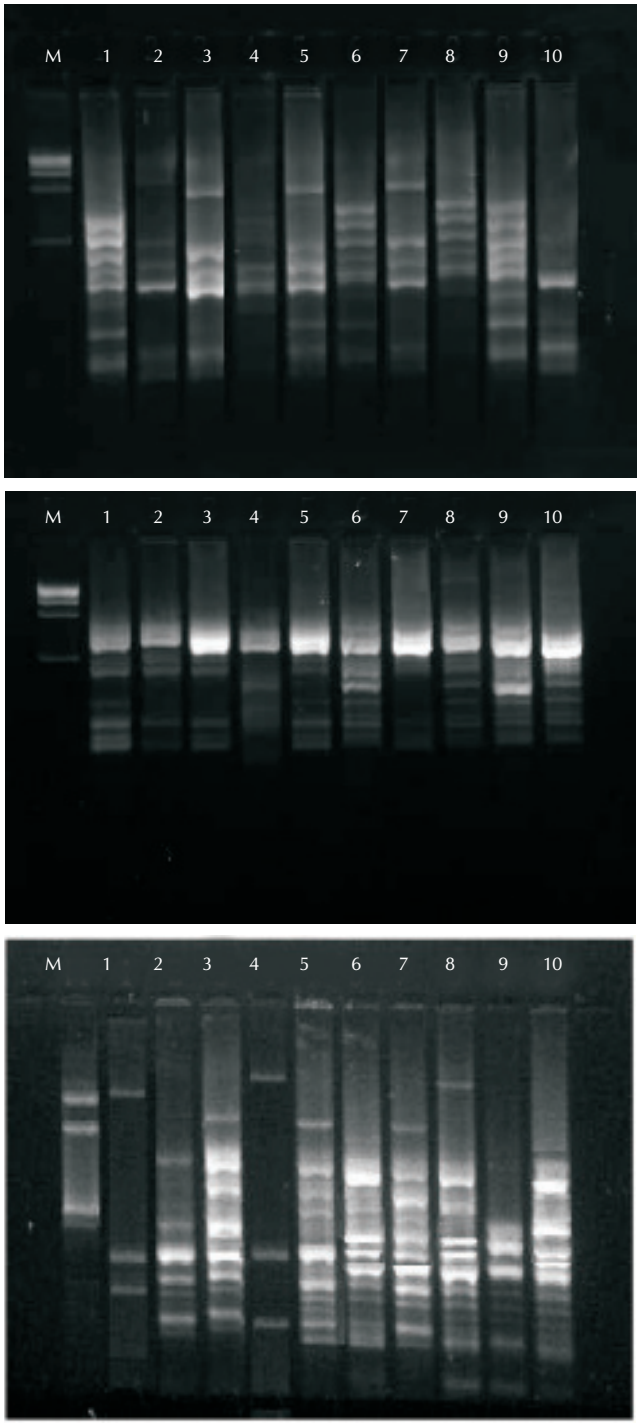

Figure 2: Figures showing amplification pattern of ten isolates of *C. falcatum* using ISSR primers UBC-850, UBC-857 and UBC- 873

(Beniwal *et al*. 1989; Satyavir *et al*. 2001).

Khirbat *et al*. (1980) evaluated five isolates of red rot pathogen and observed that strain-3 appears to be new strain because it produced susceptible reaction on clone CoL 9 and Co 62399 whereas other strains produced resistance reaction on above clones. The pathogenic variability in different isolates of *C. falcatum* Went. showed different pathogenic behaviour of the isolates on different host Beniwal *et al*. 1989. Similar findings were quoted by Ram Ji Lal *et al*. (1989) that the different isolates of *C. falcatum* Went. gave various degree of resistance or susceptible reaction on different varieties of sugarcane.

ISSR markers are useful for studying genetic diversity in *Colletotrichum* species. These are rapid, reproducible and produce a large number of polymorphic bands and aid the understanding of pathogen population dynamics, which can facilitate the development of effective control strategies. The genetic diversity of *C. falacatum* based on ISSR markers showed a correlation between genetic and geographical distribution which was supported by Ratanacherdchai *et al*. (2010) who reported that *C. capsici* isolates collected in Australia from strawberry and papaya formed a subcluster distinct from isolates from Thailand.

The intra and inter specific polymorphism among fungal pathogen that cause wilt and root rot on chick pea were investigated by using 30 RAPD and 20 ISSR primers. According to ISSR analysis, primers $(GA)_{8T}$, $(AG)_{8G}$ and $(AC)_{8T}$ produced amplification profiles that differentiated each fungal species of *Fusarium* (Bayraktar and Sara Dolar, 2007).

Similarly Kumar *et al*. (2010) utilized nine ISSR markers out of which two ISSR markers, ISSR 02 (ACTG4) and ISSR10 (CAC5) differentiated all the 25 *C. falcatum* isolates with alleles ranging from 250 to 3500 bp. The average number of bands per primer was eight with 100% polymorphism. ISSR 10 performed better in all the marker efficiency parameters.

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