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SHORT COMMUNICATION

Isolation and pathogenic variability of *Colletotrichum falcatum* causing red rot in sugarcane

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Abstract Red rot of sugarcane caused by the fungus *Colletotrichum falcatum* Went is the most destructive and severe disease worldwide. The present study identifies the *C. falcatum* which causes sugarcane red rot in the south Gujarat province of India. A survey was carried out in different sugarcane cultivars region, and nine strains isolated were identified as *C. falcatum* by using the sequencing of ITS rRNA gene. Pathogenicity tests validated on nine different sugarcane cultivars showed strain cfCHA infected maximum seven cultivars (virulence frequency 77.9 %). Interestingly, none of the *C. falcatum* isolates were able to infect the Co94004 cultivars, whereas Co671 cultivars infected by all the isolates of *C. falcatum*. This result has important implications for sugarcane breeding programmes in which Co94004 can be recommended to the farmers of south Gujarat regions, India.

Keywords Sugarcane · Red rot · *Colletotrichum falcatum* · ITS · Phylogeny

Sugarcane (*Saccharum officinarum* L.) belonging to the family *Poaceae* is an important cash crop for the farmers. In India, sugarcane cultivated in more than 3.5 million ha land and producing more than 26 million tons cane [14]. It is the second largest crop under cultivation after cotton and used as the main source of sugar and bioethanol production. Sugarcane is susceptible to many fungal diseases. Among them, red rot of sugarcane caused by *Colletotrichum falcatum* is the major constraint for sugarcane production and entire cane breeding is focused around this disease. The red rot is the chief constriction for sugarcane production, and it is responsible for the elimination of several elite clones from the field due to the continuous evolution of the newer races [10].

The red rot disease is not only prevalent in the east coast zone and north central/west part of India; it has also spread to the peninsular part like Gujarat [2]. Resistant varieties of sugarcane are important means of control against several diseases like red rot [15, 17]. Hence, systematic screening of red rot-resistant cultivars is necessary to mitigate the threat posed by *C. falcatum*. However, the newly released cultivars surrender to the pathogen almost as soon as they become popular due to the frequent emergence of new variants of the pathogen because of factors such as mutation, heterokaryosis, hybridization and adaptation [7, 9].

South Gujarat is predominately sugarcane farming area, but information is not available on pathotypes of *C. falcatum* prevalence. The study of virulence of the causal organism in its prevalent area is very important for recommending resistant varieties of the crop to the farmers. The present research aims to (1) collect red rot infected sugarcanes samples from the field and isolate the *C. falcatum*, and (2) analyse morphology and pathogenicity of pathogens in different cultivars of sugarcane.

Red rot infected canes of different sugarcane varieties showing well-developed infection were collected from the different places of south Gujarat region, India. The split canes with red, brownish black region were used to isolate the organism in laboratory condition. Briefly, the infected

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parts of the internodal tissues were cut into small pieces, washed with sterile distilled water to become clear. Surface sterilization was carried out with 0.1 % mercuric chloride ($HgCl_2$) solution for 30 s, followed by three consecutive washes with sterile distilled water. Sample pieces were allowed to dry and transferred aseptically to potato dextrose agar (PDA) medium. These Petri dishes were incubated at 28 ± 2 °C in the incubator until the mycelial growth initiation. White mycelia from the growing edge were subcultured aseptically on fresh PDA plates. The pure culture was microscopically examined for its purity and further purified by repeated subculture using the hyphal tip method of isolation. The pure culture was maintained on PDA slants at 4 °C for further study.

The morphological characters such as conidia and setae of all isolates were studied on PDA. Twenty-five conidia and setae for each isolate were assessed for morphometric measurements under a light microscope at 10× and 40× using a micrometry. For molecular identification, total genomic DNA was extracted and purified according to the method adopted by Raeder and Broda [12]. The internal transcribed spacer (ITS) regions were amplified with the universal primers ITS1 and ITS4 [19]. Briefly, PCRs were carried out on a thermal cycler (Eppendorf) in 50 µl containing 10× PCR buffer, 20 ng of DNA, 2.5 mM (each) dNTPs, 1 µM each primer and 1U of *Taq* polymerase (Merck, Bengaluru, India). The amplifications were performed as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 1 min, extension at 72 °C for 2 min and final extension for 10 min at 72 °C. The amplified products of PCR were resolved on 1.5 % (w/v) agarose gel. The PCR products spanning approximately 600 bp were sent for sequencing to Bioinnovations, Mumbai. ITS gene sequence of the isolate was compared with ITS sequences available in the BLAST search in National Centre for Biotechnology Information, GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) by performing BLASTn search. The sequences were submitted to GenBank under the following accession numbers: KP869830–KP869834 and KP205441–KP205444 (09 sequences).

For pathogenicity assay under field condition, nine sugarcane cultivars such as Co86249, Co86002, Co671, Co671, Co86032, Co94004, Co5071, CoM265, Co8145 and Co5072 were used to study the pathogenic variability of *C. falcatum*. Ten canes of each variety, free from insect–pest and disease, were inoculated by standard plug method [16]. Inoculations were made in the middle of the third exposed internodes, from the bottom. The bore holes were immediately sealed with plastic tap to avoid contamination, and inoculated canes were labelled. After 2 months of incubation, the inoculated canes were split open longitudinally along the point of inoculation and graded on the basis of international scale 0–9. Disease reactions were scored on the basis of differential disease index as R: Resistant (0–2), MR: Moderately Resistant (2.1–4.0), MS: Moderately Susceptible (4.1–6.0), S: Susceptible (6.1–8.0) and HS: Highly Susceptible (>8.0) groups of the host [18].

Morphological characterization, including colony morphology, conidial and setae size, was performed. On the basis of mycelial growth pattern on PDA, isolates could be categorized into two groups: P1 group (cfNAV, cfVES and cfPAR) with dense mycelia with concentric ring of orange-coloured spores and P2 group (cfTIM, cfMAR, cfGAN, cfKAM, cfCHA and cfMAD) with sparse mycelia with dispersed blackish to orange-coloured spores. The strains showed higher variability of size of conidia and setae (Table 1). The isolate cfKAM (20.0 × 5.09 µm) showed smallest and cfPAR (25.52 × 5.33 µm) largest conidia, whereas in setae, cfNAV (112.37 × 4.50 µm) had the smallest and cfTIM (167.66 × 4.50 µm) had the largest setae. Colony morphology ranged from white mycelium to grey mycelium, the isolate cfMAR showed maximum growth at 7 days, whereas cfNAV showed minimum

Table 1 Cultural and morphological characters of *C. falcatum*

Isolates	Cultural character		Conidia (µm)**		Setae (µm)**	
	Mycelial growth on PDA (cm)*	Colour	Length	Width	Length	Width
cfNAV	5.86 ± 0.05	Light grey	20.31–28.03	4.63–6.23	83.24–138.32	3.87–5.32
cfVES	6.01 ± 0.20	Light grey	21.75–26.66	3.2–5.12	101.74–159.34	2.58–5.16
CfPAR	6.43 ± 0.05	Dull white	23.03–29.51	4.63–5.83	124.05–174.31	5.32–9.41
cfTIM	7.73 ± 0.05	White	19.48–26.09	3.56–6.23	138.61–192.19	2.89–6.45
cfMAR	8.00 ± 0.10	Dull white	15.87–26.14	2.75–5.57	133.51–201.10	1.29–4.65
cfGAN	7.86 ± 0.15	White	22.45–29.58	3.88–5.76	137.87–157.23	3.56–8.26
cfKAM	7.76 ± 0.05	White	18.94–21.92	3.25–6.05	132.33–167.29	2.58–8.16
cfCHA	7.03 ± 0.45	White	17.23–28.92	4.41–5.71	139.67–214.67	6.73–8.66
cfMAD	7.73 ± 0.25	White	23.58–26.55	4.81–5.97	134.90–156.94	3.44–5.87

* Mean value of five replicates after 7 days of inoculation; ** Data represent the mean of 25 microscopic fields

growth (Table 1). The present study showed high variability of *C. falcatum* with different morphological characters belonging to the same species which is in good agreement with the observation reported by many researchers [1, 3, 5, 6, 11].

Sequence analysis of ITS1, 5.8S RNA gene and ITS2 was determined for 09 isolates to confirm the species identity. The phylogenetic analyses was carried out on the basis of ITS region in MEGA5 using the neighbour-joining method. The phylogeny of ITS1 sequence reveals the genetic divergence among the nine *C. falcatum* isolates [11]. Phylogenetic analysis grouped all the isolates into six clusters (Sup. 1). Morphologically dissimilar isolates cfVES and cfCHA fall into cluster one, but isolates cfKAM and cfMAD (P2 morphology group) present together into same cluster. This reveals that there would not perfect correlation between morphological and molecular characters. *C. falcatum* isolates cfMAD collected from Co86249 and cfKAM collected from Co92004 fall into cluster two in a well-supported subclade with high (82 %) bootstrap value. Notably, CoC671 isolate cfNAV and Co86002 isolate cfTIM fall into cluster three along with Co1148 isolate cf01 (NCBI accession KU220959). Out of nine, three isolates, namely cfPAR, cfMAR and cfGAN, could not fall into any cluster, which indicates that they could be more varied in ITS regions. Isolate cfGAN from Co86032 was highly divergent from all other isolates and present on a separate clade.

Pathogenic variability of the nine isolates on a set of nine different cultivars revealed that the isolate cfCHA infected maximum of seven (77.8 %) cultivars followed by cfMAD (55.5 %), cfKAM (55.5 %), cfNAV (44.4 %) and cfPAR (44.4 %), respectively, were found to be more virulent, whereas cfGAN was least virulent (11.1 %) infecting only one cultivar (Tables 2 and 3). Frequency of susceptibility of different sugarcane cultivars showed none of the isolates were able to infect Co94004 cultivar, whereas Co671 was more susceptible (Table 4). Morphological studies of *C. falcatum* such as growth, sporulation and conidial germination had negative correlation with the frequency of infecting the sugarcane cultivars. Pathogenicity studies showed that the behaviour of *C. falcatum* pathotypes significantly varied in response to host resistance and it was negatively correlated morphological characters. The results are in good agreement with the study carried out by Malathi et al. [9]. Pathogenicity tests divided the pathogenic potential of *C. falcatum* into low-, medium- and high-virulence groups. It clearly revealed that

Table 2 Pathogenic behaviour *C. falcatum* isolates on differential hosts

Isolates	Sugarcane varieties								
	Co86249	Co86002	Co671	Co86032	Co94004	Co5071	CoM265	Co8145	Co5072
cfNAV	MR	MR	HS	MS	R	MR	MS	MR	HS
cfVES	MR	R	HS	HS	R	R	MR	MR	S
cfPAR	R	R	HS	HS	MR	MS	MR	MR	MS
cfTIM	MS	MR	HS	MR	R	R	MR	MR	MR
cfMAR	MR	MS	HS	R	R	MR	MR	MS	MR
cfGAN	R	MR	HS	MR	R	MR	R	MR	MR
cfKAM	MS	S	HS	MR	R	R	MS	MR	MS
cfCHA	MS	MS	HS	MS	R	MS	MS	S	MR
cfMAD	MS	MS	HS	MR	MR	MR	R	MS	MS

R resistant, *MR* moderately resistant, *MS* moderately susceptible, *S* susceptible, *HS* highly susceptible

Table 3 Frequency of virulence of *C. falcatum* on sugarcane differential hosts

Sr. no	Isolates	No. of differential host infected	Virulence frequency (%)
1	cfNAV	4	44.4
2	cfVES	3	33.3
3	cfPAR	4	44.4
4	cfTIM	2	22.2
5	cfMAR	3	33.3
6	cfGAN	1	11.1
7	cfKAM	5	55.5
8	cfCHA	7	77.8
9	cfMAD	5	55.5

Table 4 Frequency of susceptibility of different sugarcane varieties to nine *C. falcatum* isolates

Sr. no	Sugarcane variety	No. of virulent isolates	Susceptibility (%)
1	Co86249	4	44.4
2	Co86002	4	44.4
3	Co671	9	100
4	Co86032	4	44.4
5	Co94004	0	0
6	Co5071	2	22.2
7	CoM 265	3	33.3
8	Co8145	3	33.3
9	Co5072	5	55.5

nine isolates of *C. falcatum* inoculated on nine sugarcane differentials Co671 are the more susceptible (Sup. 2) and Co94004 more resistant cultivars. Pathogenicity behaviour is supported by the earlier studies [8, 13]. It indicated that phytopathogenic organisms are constantly subjected to extinction and re-colonization and are rarely found in equilibrium. The process of co-evolution resulting from selection pressure exerted by the plant, and the pathogen is considered to be potential mechanism acting on virulence diversity [4].

The present study information is critical for devising management strategies, selection and development of resistant cultivars. The high level of variability of the *C. falcatum* makes it difficult to breed for red rot resistance. The use of genetic resistance as a method to control the disease economically is of great interest, and therefore, information about the variability of the fungus in each region is the basis for resistance breeding programmes.

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