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PATHOLOGICAL AND MOLECULAR VARIATION IN *COLLETOTRICHUM FALCATUM* WENT ISOLATES CAUSING RED ROT OF SUGARCANE IN THE NORTHWEST ZONE OF INDIA

Pratigya Saksena, Sunil Kumar Vishwakarma, Ajay Kumar Tiwari, Atul Singh, Arun Kumar*

Uttar Pradesh Council of Sugarcane Research
Shahjahanpur-242001, Uttar Pradesh, India

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Abstract: Red rot is one of the most wide spread sugarcane diseases in the country and has been a constraint on sugarcane productivity. Pathological as well as molecular studies were used to characterize the 11 isolates of *Colletotrichum falcatum* Went collected from sugarcane cultivars of different sugarcane-growing regions in northwestern states of India, to assess pathogen diversity. Seven reference pathotypes of *C. falcatum* from the northwestern zone of India were compared with four newly collected isolates of the same pathogen. All the newly collected isolates and existing pathotypes were inoculated on a set of 14 differentials in August 2011 by the plug method. After 60 days of inoculation, the observations were recorded and the pathotypes/isolates were categorized as resistant, intermediate, and susceptible according to the virulency behavior. On the basis of pathological categorization and comparison with reference pathotypes, it was concluded that the three isolates R1001 (CoJ 64), R1002 (CoS 88230), and R1004 (CoSe 92423) are similar to the existing isolate Cf 08, except for isolate R0401 from CoS 8436 (Shahjahanpur, Uttar Pradesh). This isolate differs from all the reference pathotypes of the northwestern zone of India indicating the existence of a new pathotype. Pathological results revealed that variety CoJ 64 is the ancestor/source of prevailing new races in nature because these three new isolates showed similarity with Cf 08, of CoJ 64. In this area, Cf 08 was widespread. The isolates were further tested for their variability with random amplification of polymorphic DNA (RAPD) primers. Twenty RAPD primers were screened, out of which seven gave amplification. Out of seven amplified primers, only two primers showed the polymorphism among 11 isolates (seven reference pathotypes and four new isolates) of *C. falcatum.* Analysis of the genetic coefficient matrix derived from the scores of RAPD profiles showed that minimum and maximum per cent similarities among the tested *C. falcatum* isolates existed in the range of 11.11 to 87.5, respectively. The dendogram analysis by the unweighted pair group method with arithmetic mean (UPGMA), separated two main clusters. The first cluster comprises only two isolates (Cf 07 & Cf 08), however the second cluster comprises all the other isolates (Cf 01, Cf 02, Cf 03, Cf 09, Cf 11, R1001, R1002, R1004 and R0401), confirming high genetic diversity among the isolates. The study also indicates the possibilities of a new isolate (R0401) in Shahjahanpur, which needs further investigation at the sequence level. The investigation is in progress.

Key words: *Colletotrichum falcatum,* pathological categorization, polymorphism, RAPD

INTRODUCTION

Sugarcane is an economically important crop which is vegetatively propagated in the tropics and sub-tropics. In India, sugarcane is also an important cash crop used as the chief source of sugar and for ethanol production (as a renewable source of bio-fuel). Efforts for providing protection from red rot are equally important in the new strategies for improving cane and sugar productivity. The loss in cane weight was recorded to be about 29.07% and resulted in a 30.8% loss in sugar recovery by red rot (Hussnain and Afghan 2006). Red rot disease is caused by the fungus *Colletotrichum falcatum* Went [Perfect stage; *Glomerella tucumanensis* (Speg. Von Arx and Muller)]. The disease is also responsible for the deterioration of sugarcane cultivars. Red rot disease continues to be a problem in other countries such as the USA, Bangladesh, India, Australia, Thailand, and Taiwan. The worldwide loss in cane yield and in sugar recovery was about 5–10% (Viswanathan and Samiyappan 2002). Severe damage to crop stand has been found in many varieties due to disease epidemics in the states of Haryana, Uttar Pradesh, and Bihar. The disease was first identified by Went in Java (1893) and Chona (1954) with special reference to India. Red rot occurs in various parts of the cane plant but it is usually considered as a stalk and a seed-piece disease (Alvi *et al.* 2008). Red rot disease caused by *C. falcatum* is one of the most serious threats to sugarcane cultivation in India (Beniwal *et al.* 1989; Alexander and Viswanathan 1996; Freeman 1997; Duttamajumdar 2008). In India, the disease is highly destructive in the northwestern part of the country due to the high humidity and temperature in that area (Tiwari *et al.* 2010). However, it has also spread to the peninsu-

*Corresponding address:
ajay_biotech2005@rediffmail.com

lar parts of the country. Symptoms of red rot are highly variable depending upon the susceptibility of the sugarcane cultivar, pathogen virulency, and environment. The pathogen shows a great diversity in virulency as numerous isolates are known to occur in nature which have been classified on the basis of host differential reaction (Singh and Lal 1999; Abbas *et al*. 2010; Singh *et al.* 2012).

The pathogen spreads primarily through infected sugarcane setts and hence the use of disease free planting materials is essential for preventing disease development in the field (Velazhahan *et al.* 2012). A recent study regarding red rot was reported by Kumar *et al.* (2010) and Karunanithi *et al.* (2012) on pathological and molecular variation. There are many reports of genetic characterization in other species of Colletotrichum, but much less information is available on the relative importance of *C. falcatum* pathotype races – their distribution and diversity (Kumar *et al.* 2010). Variability in *C. falcatum* isolates have been characterized by using morphological characters, host reaction, and an arbitrary marker system such as RAPD and ITS primers (Madan *et al*. 2000; Mohan Raj *et al*. 2002; Suman *et al*. 2005; Singh *et al*. 2012). The aim of the present investigation was to characterize more isolates which prevail in Uttar Pradesh and to study the molecular as well as the pathological variation of *C. falcatum*, for the possible management of the disease.

MATERIALS AND METHODS

Collection, isolation and maintenance of the isolates

Four isolates viz; R1001 (CoJ 64), R1002 (CoS 88230), R1004 (CoSe 92423), and R0401 (CoS 8436) were isolated from the red rot infected stalks of different regions in Uttar Pradesh (Table 1). The collected stalks were surface sterilized, and the infected parts of the internodal tissues were taken out with the help of a cork borer. These parts were placed on Oat Meal Agar (OMA) in petri-dishes under aseptic conditions and maintained at 30±2°C until the mycelial growth was initiated. These cultures were further purified by a regular transfer to the fresh OMA medium and then incubated at 30±2°C until sporulation.

Table 1. *C. falcatum* Went isolates of sugarcane used in this study and their place of collection

No.	Isolates	Place of collection	States in India
1	Cf 01	Yamuna Nagar	Haryana
2	Cf 02	Karnal	Haryana
3	Cf 03	–	Haryana
4	Cf 07	Naraingarh	Haryana
5	Cf 08	Bhatinda	Punjab
6	Cf 09	Kaithal	Haryana
7	Cf 11	–	–
8	R0401	Shahjahanpur	Uttar Pradesh
9	R1001	Shahjahanpur	Uttar Pradesh
10	R1002	Nawabganj	Uttar Pradesh
11	R1004	Palia	Uttar Pradesh

Pathological assay

In the month of August, the inoculation of these isolates was done on the 14 differentials viz. Co 419, Co 975, Co 997, Co 1148, Co 7717, Co 62399, CoC 671, CoJ 64, CoS 767, CoS 8436, BO 91, Baragua, Kakhai, SES 594. These differentials were inoculated by the plug method with seven characterized pathotypes and four new isolates, for grading their resistance behavior.

Molecular assay

DNA isolation

Fungal DNA was extracted from 0.5 g of fresh mycelial mat using a cetyl tri methyl ammonium bromide (CTAB) protocol (Saghai-Maroof *et al.* 1984) with the modification, that in place of lyophilized fungal mat in the original protocol, fresh mycelial mat was ground in liquid nitrogen. The DNA concentration was determined through agarose-gel electrophoresis using a known concentration of k-uncut DNA as the standard. The purified DNA was quantified by running 2 µl of the sample on a 0.8% agarose gel and to adjust the final concentration of the template to 20–25 ng/2 µl for use in polymerase chain reaction (PCR) analysis. The different components of PCR were optimized to get appropriate amplification. For the 25 µl reaction mixture, Taq Polymerase (0.1 µl), dNTPs (0.2 µl), Taq buffer (2.5 µl MgCl2 added), RAPD Primer (l µl), DNA (2 µl), and DDW (19.2 µl) were used.

PCR amplification

Amplification was performed using the following temperature profile: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 35°C for 2 min, extension at 72°C for 2 min, and final extension at 72°C for 5 min. Steps 2–4 were carried out using RAPD primers according to Saiki *et al.* (1988). A set of 20 RAPD primers were used as presented in table 2. The amplified products were subjected to agarose gel electrophoresis using 1.5% agarose and the gel was analyzed on a gel documentation system.

Table 2. RAPD primers and their sequences

No.	Primer code	Primer sequence 5′–3′
1	A2	TGCCGAGCTG
2	A3	AGTCAGCCAC
3	A4	AATCGGGCTG
4	A5	AGGGGTCTTG
5	A6	GGTCCCTGAC
6	A7	GAAACGGGTG
7	A8	GTGACGTAGG
8	A9	GGGTAACGCC
9	A10	GTGATCGCAG
10	AJ-01	ACGGGTCAGA
11	AJ-02	TCGCACAGTC
12	AJ-03	AGCACCTCGT
13	AJ-04	GAATGCGACC
14	AK-01	TCTGCTACGG
15	AK-02	CCATCGGAGG
16	AK-03	GGTCCTACCA
17	AK-04	AGGGTCGGTC
18	AK-06	TCACGTCCCT
19	AK-08	CCGAAGGGTG
20	AK-09	AGGTCGGCGT

RESULTS

Pathogenicity assay

The isolates varied considerably with respect to the pathogenic reaction of 11 isolates on 14 differentials according to the All India Coordinated Research Project (AICRP). Various symptoms were noticed. The common characteristic symptom was, discolouration and yellowing of the young crown leaves. The discolouration and withering continued from the tip to the leaf base until the whole crown withered. The plant died within 10–15 days. For calculating the disease index, observations were recorded on the nature of white spots, nodal transgression, lesion width, and condition of the tops, by splitting the canes open after 60 days of inoculation as per AICRP norms. The isolates showed the resistant, susceptible, and intermediate reactions on the differentials. It was found that among four new isolates, there were three isolates: R1001, R1002, R1004 which expressed a similar reaction as isolate Cf 08. However, one isolate (R0401) from CoS 8436 differed from the other three new isolates on the basis of pathogenic behavior. This difference indicated the existence of new pathotypes (Table 3). These three new collected isolates, were found to be more virulent, infecting 8 varieties among 14 used in this study. Least virulent were Cf 03 and Cf 11, infecting only 3 varieties. Such results might reflect the widely spreading nature of the pathotype Cf 08 in this area. It may also be concluded that the isolate Cf 08 is more harmful for newly developed varieties.

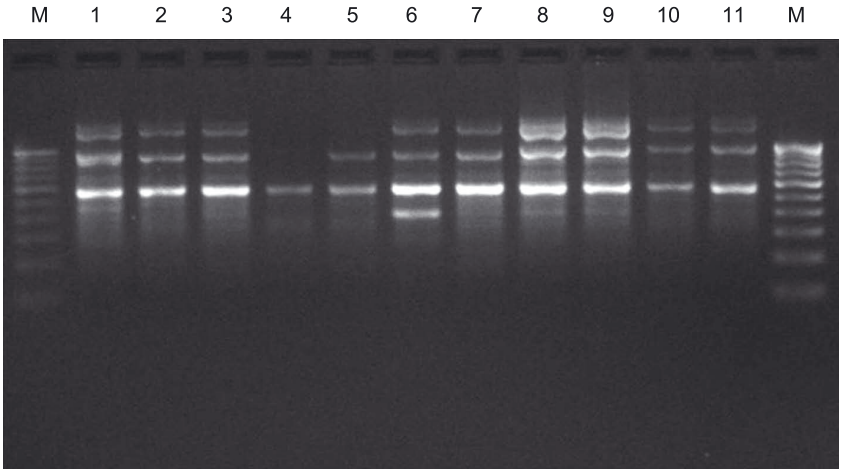
Table 3. The pathogenic behavior of *C. falcatum* pathotypes/isolates on host differentials

Isolates	Host	Reaction on host differentials													
		Co 419	Co 975	Co 997	Co 1148	Co 7717	Co 62399	CoC 671	CoJ 64	CoS 767	CoS 8436	BO 91	Barag-ua	Kak-hai	SES 594
Cf 01	Co 1148	R	S	S	S	R	S	S	S	R	R	R	R	S	R
Cf 02	Co 7717	I	R	S	R	S	I	S	I	R	R	R	R	S	R
Cf 03	CoJ 64	R	R	S	R	R	R	I	S	R	R	R	R	S	R
Cf 07	CoJ 64	I	R	S	S	R	R	I	S	R	R	R	R	S	R
Cf 08	CoJ 64	I	S	S	S	S	S	S	S	I	R	R	R	S	R
Cf 09	CoS 767	I	I	S	S	R	R	I	S	S	R	R	R	S	R
Cf 11	CoJ 64	S	I	S	I	I	I	I	S	I	R	I	I	I	R
R0401	CoS 8436	S	S	S	I	R	S	S	S	R	S	R	R	S	R
R1001	CoJ 64	I	S	S	S	S	S	S	S	I	R	R	R	S	R
R1002	CoS 88230	I	S	S	S	S	S	S	S	I	R	R	R	S	R
R1004	CoSe 92423	I	S	S	S	S	S	S	S	I	R	R	R	S	R

R – resistant, S – susceptible, I – intermediate

PCR assays

The presence of *C. falcatum* was demonstrated by electrophoresing the PCR products on agrose gel. The eleven isolates of *C. falcatum* were characterized by RAPD-PCR analysis. Out of the 20 primers used, 7 showed amplification (Fig. 1). The seven primers used for RAPD analysis were A3, A4, A9, AJ2, AJ3, AK1, and AK2. Primer A3 and A9 amplified a total of 78 fragments in eleven isolates of *C. falcatum*, whereas primer AJ2 amplified only 7 fragments. Thus in the present set of genotypes, primer A3 and A9 were most informative. Analysis of the genetic coefficient matrix derived from the scores of RAPD profiles, showed that minimum and maximum per cent similarities among the tested *C. falcatum* isolates were in the range of 11.11 to 87.5, respectively (Fig. 2). The dendogram analysis by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), separated two main clusters. The first cluster grouped only two pathotypes/isolates (Cf 07 & Cf 08), however, the second cluster included all the other pathotypes/isolates (Cf 01, Cf 02, Cf 03, Cf 09, Cf 11, R1001, R1002, R1004 and R0401) confirming high genetic diversity among the isolates. The isolate Cf 02 was more diverse from all other isolates. There were two subclusters, 1 and 2. The first subcluster was composed of Cf 01, Cf 03, Cf 09, and Cf 11, while the second subcluster grouped all four new isolates R1001, R1002, R1004, R0401, indicating the close relation among them. Cf 03–Cf 01, Cf 09–Cf 11 showed more similarity. A similar trend was seen in R1001–R1002 and R0401–R1004 isolates. The dendogram analysis clearly indicated the genetic diversity of the *C falcatum* isolates collected from the different regions of the Uttar Pradesh.



Lane M – Marker (100 bp DNA Ladder), L1 – Cf 01, L2 – Cf 02, L3 – Cf 03, L4 – Cf 07, L5 – Cf 08, L6 – Cf 09, L7 – Cf 11, L8 – R10 01, L9 – R10 02, L10 – R10 04, L11 – R0401 isolates/pathotypes of *C. falcatum*

Fig. 1. Isolates of *C. falcatum* showing amplification with RAPD Primers as--

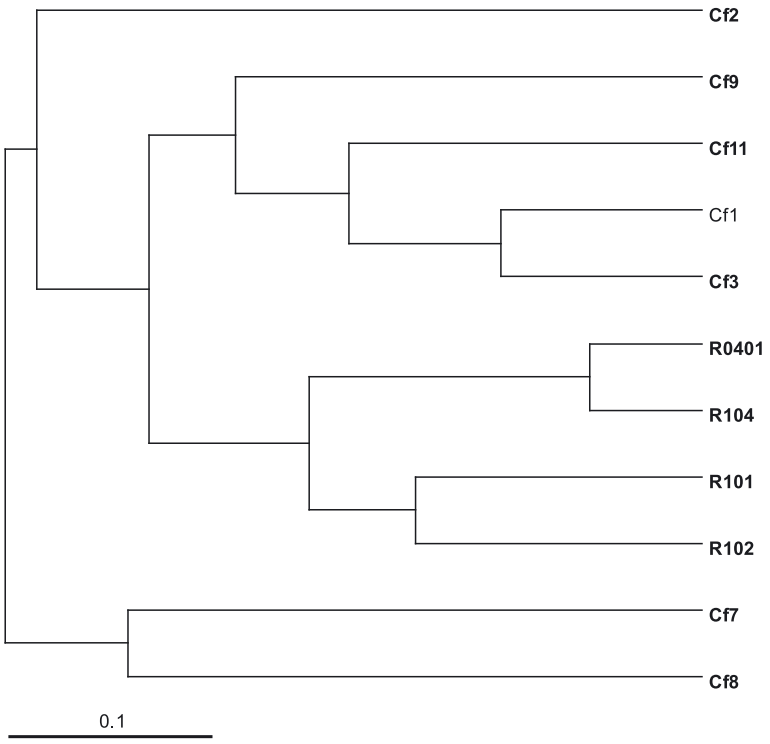


Fig. 2. UPGMA based dendogram showing molecular diversity among different isolates of *C. falcatum*

DISCUSSION

Different types of symptoms were recorded on different varieties. The most common symptom observed in those fields affected by red rot, was discoloration and yellowing of the young crown leaves. The discoloration and withering continued from the tip to the leaf base until the whole crown withered. The plant died within 10–15 days. According to the disease index, it was found that among four new isolates, R1001, R1002, R1004 showed a similar reaction with existing pathotype Cf 08. Such a reaction might be due to the widely spreading nature of the pathotypes Cf 08 in the study area. It may also be concluded that CoJ 64 (Cf 08) will be more harmful for newly developed varieties. A different reaction was shown by R0401 isolated from CoS 8436 indicating that R0401 is a new isolate. Agnihotri (1990) suggested that the production of new strains in *C. falcatum* by hybridization could not be ruled out beside mutation. Under favorable conditions, the disease spreads quickly in the susceptible varieties. The rapid evolution of *C falcatum* due to the planting of susceptible varieties which result in the emergence of new virulent strains, causes a breakdown in the varietal resistance (Mishra and Behera 2009; Muhammad *et al.* 2011).

Kumar *et al.* (2010), in their investigation, showed the high molecular diversity of the red rot isolates (Cf 01, 02, 03, 07, 08, 09) through the RAPD, URP, and ISSR markers from

different commercials varieties grown in north India. The existence of the new isolate R0401, needs further investigation at the sequence level. However, Singh *et al.* (2012) recently generated the sequence level information of three red rot isolates and showed the high genetic variability of the isolates causing heavy yield losses in eastern Uttar Pradesh. According to Jain and Chahal (2011), the geographical origins of the isolates could not be correlated with molecular and pathological diversity, since one of the most efficient ways to prevent this devastating disease is through the use of pathogen-free planting materials in commercial production.

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