Chapter 5

Molecular identification and genetic diversity of *Colletotrichum falcatum* isolates

Introduction

Red rot disease of sugarcane caused by the fungus *Colletotrichum falcatum* Went. (teleomorph, *Glomerella tucumanensis* (Speg.) Arx and E. Müller), is the most destructive disease of sugarcane worldwide. It poses a major threat in successful cultivation of sugarcane (Satyavir, 2003; Duttamajumder, 2008; Viswanathan and Rao, 2011). Red rot epidemics cause significant annual losses of sugarcane yields that may reach up to 100% under conditions favourable for disease development. Several epiphytotics of red rot have resulted in failure of many important Indian commercial sugarcane varieties (Singh, 2008). The pathogen shows a great diversity in virulence as a number of pathotypes are known to occur in nature. Ten races of *C. falcatum* have been reported in India based on host differentials (Singh, 2008).

Being a long duration crop, sugarcane faces several problems such as biotic and abiotic stresses. Among biotic factors, red rot disease caused by fungus *C. falcatum* is the most important. This disease has been responsible for the failure or deterioration of many popular varieties. The red rot threat is still continuing as a major problem in several sugarcane growing countries such as USA, Bangladesh, India, Australia, Thailand and Taiwan (Alexander and Vishwanathan, 2002).

In India, the disease is highly destructive in the north-western part of the country due to prevalence of high humidity and temperature. However it has spread to the peninsular parts also. It infects various parts of the
sugarcane plant but stalk is more vulnerable. Therefore it is considerable a stalk and seed plant disease (Suman et al., 2005). Symptoms of red rot are highly variable depending upon the susceptibility of sugarcane cultivar, pathogen virulence and environment. The pathogen shows a great diversity in virulence as numerous isolates are known to occur in nature which has been classified on the basis of host differential reaction (Singh and Lal, 1999).

In the subtropical plains of India, monsoon period in the months of June–July make Saccharum genotypes very vulnerable to the attack of C. falcatum, resulting in complete devastation of the standing crop. Several isolates of C. falcatum have been isolated from diseased plant samples, of which 6 promising pathotypes, prevalent in northwestern subtropical part of India, have been identified on the basis of their pathogenic behavior on different hosts. To date, these pathotypes have been differentiated on the basis of morphology, physiology and host reactivity parameters. However, no sufficient information on their differentiation at genetic level is available for the existing pathotypes (Suman et al., 2005; Kumar et al., 2010). Further, no much efforts have been made to work out their genetic diversity by sequencing of PCR products. Therefore, the present study was undertaken to characterize three red rot isolates collected from different parts of Uttar Pradesh using specific ITS primers. The ~550 bp amplicon from three isolates was cloned, sequenced and consensus sequences were submitted in GenBank. The sequence data of ITS region of these isolates were analyzed for their identification based on sequence identities, diversity and phylogenetic relationship using the similar sequence data of selected red rot isolates reported across the world.
Review of Literature

Alexander et al. (1985) and Beniwal et al. (1989) classified *C. falcum* isolates in India into different pathotypes based on their differential reaction. Subsequent workers reported the existence of different pathotypes of *C. falcum* using host differentials (Kumar and Virk, 2001; Nageswararao and Achutaramarao, 2004; Nageswararao and Patro, 2005). On the basis of these host differentials, Satyavir (2003) summarized Cf 01, Cf 02, and Cf 03 in varieties Co 1148, Co 7717, and CoJ 64, respectively from the North West Zone and Cf 04, Cf 05, and Cf 06 pathotypes in varieties Co 419, Co 997, and CoC 671, respectively from East Coast Zone. Subsequent studies on pathogenic variability during 1993-2000 revealed the existence of four new pathotypes viz., Cf 07, Cf 08, Cf 09, and Cf 10. But all these pathotypes except Cf 09 were virulent on CoS 767. The breakdown of resistance in this cultivar was noticed in Haryana and U.P. in recent years and these studies confirmed the appearance of a new pathotype (Cf 09) capable of breakdown of resistance of this widely cultivated cultivar in North West Zone (Satyavir et al., 2001). Madan et al. (2000) studied the molecular variability of five different isolates of *C. falcum* from Haryana through RAPD-PCR technique using five decanucleotide primers and grouped the isolates into two genetic clusters. Mohanraj et al. (2002) used 80 random primers in RAPD based studies and categorised the isolates of *C. falcum* into four different groups i.e. Gr-1 (Co 7717); Gr-2 (Co 1148); Gr-3 (CoC 671, CoC 92061, CoC 85061), and Gr-4 (CoC 90063, CoS 767).

Suman et al. (2004) recorded a high degree of polymorphism (78.6 %) and more than 50 % genetic divergence among the pathotypes. Unweighted pair group method analysis (UPGMA) of genetic similarity indices grouped the six pathotypes into two clusters; Cluster-1 comprised of Cf 01 and Cf 09 while cluster-II comprised the remaining four pathotypes. Wijesekara et al. (2005) examined 20 *Colletotrichum* isolates from 14 different crops including sugarcane using 16 primers in RAPD
technique and sugarcane isolates 4800 and 4803 produced an identical banding pattern while difference existed in their morphological characters.

Over the past few decades, immunological and molecular diagnostic methods have increasingly received attention as an alternative or complement to conventional methods (Schaad et al., 2003). Molecular methods based on polymerase chain reaction (PCR) are being used more frequently for detecting fungal pathogens in plant tissues (Bonants et al., 1997; Zhang et al., 1999; Kong et al., 2003; Shen et al., 2005; Wang et al., 2006), owing to increased specificity and sensitivity compared with more traditional techniques. Also, large numbers of samples can be processed in a short time by these methods. Nithya et al. (2012) identified new isolate of red rot in the Tamil Nadu regions by RAPD analysis. The RAPD primer OPE-01 amplified a ~ 560 bp fragment to from most of the C. falcatum isolates tested, regardless of the geographic origin (Nithya et al., 2012). He also developed a sequence characterised amplified region (SCAR) marker based on the RAPD data for accurate and sensitive detection of C. falcatum in infected sugarcane setts by using PCR.

Disease diagnosis and pathogen identification by conventional methods, which involve isolating the pathogen and characterizing it by inoculation tests, are labour-intensive and time-consuming. Over the past few decades, immunological and molecular diagnostic methods have increasingly received attention as an alternative or complement to conventional methods (Schaad et al., 2003). Serological methods (enzyme linked immunosorbent assay) are routinely used in several laboratories for these purposes because they allow sensitive and simultaneous analysis of many samples in a single micro plate. One major drawback of serological assays, however, is false positives caused by cross-reaction of antibodies with plant debris or unrelated organisms (De Haan et al., 2000). Molecular methods based on polymerase chain reaction (PCR) are being used more frequently for detecting fungal pathogens in plant tissues (Bonants et al., 1997; Zhang et al., 1999; Kong et al., 2003; Shen et al., 2005; Wang et al.,
2006), owing to increased specificity and sensitivity compared with more traditional techniques. Also, large numbers of samples can be processed in a short time by these methods.

The pathogen shows a great diversity in virulence as a number of pathotypes of the disease in the field as reddening of the internal tissues with interrupted red and white patches, the characteristic symptoms of the disease, develops on the stem only at later stages. Furthermore, latent infection occurs frequently making visual diagnosis impossible is known to occur in nature. Ten races of *C. falcatum* have been reported in India based on host differentials (Singh, 2008). Because of the highly variable nature of the pathogen even if a disease resistant variety is released for cultivation, it becomes susceptible to red rot disease within 8–10 years, as there is development of new more virulent races of the pathogen (Yadav, 2006). In the early stages of infection, it is difficult to recognize the presence avenues for prevention of this devastating disease. Hence, a sensitive and reliable detection technique is needed to produce pathogen-free crops.

In addition to PCR, a combination of different DNA based techniques is recently available to study variability among fungal pathogen. Ribosomal RNA (rRNA) is the most conserved (least variable) gene in all cells of living organisms. For this reason, genes that encode the rRNA (rDNA) are sequenced to identify an organism’s taxonomic group, calculate related groups, and estimate rates of species divergence. The internal transcribed spacer regions (ITS) of rDNA are non-coding and variable and the 5.8s rRNA gene, coding and conserved are valuable in determining the genetic relatedness among fungal species. Though they have low level of intra-specific variation and high inter-specific polymorphism, they have been proved to be useful tool in species confirmation of *Colletotrichum*. Malathi *et al.* (2009) conducted detailed studies on molecular analyses in a set of nine *C. falcatum* pathotypes representing tropical and subtropical regions. The results showed limited variation in 5.8s-ITS region in nucleotide sequences and they differed in
one base pair. However, phylogenetic analysis clearly showed two genetically divergent groups, which almost correspond to the tropical (clade I), and sub-tropical (clade II) conditions except for one isolate Cf7717 which belonged to tropical pathotypes clade. Studies of O’Donnell et al. (1998) and Carbone and Kohn (1999) found measuring close genealogical relationships is useful particularly for the phylogenetic examination of fungal species and the intra-specific variation is notably more abundant in such non-coding regions. In further studies, Malathi et al. (2009) used 80 C. falcatum isolates isolated from 46 cultivars prevalent all over India for molecular analyses. Phylogenetic analysis of ITS sequences identified the genetic divergence under three distinct molecular groups as Group I, II and III. Multi-locus analysis with three gene sequences of housekeeping genes like of actin, calmodulin and glyceraldehydes-3 phosphate dehydrogenase with their introns for conserved proteins among 25 isolates implicated existence of Group III and least genetic support or the movement of gene sequences between Groups I and II. Studies at Sugarcane Breeding Institute (SBI), Coimbatore revealed a clear molecular variation in C. falcatum (Viswanathan 2010).

Nithya et al. (2012) developed a polymerase chain reaction (PCR) assay for accurate and sensitive detection of C. falcatum in planting materials. Randomly amplified polymorphic DNA (RAPD) analysis identified a 566 bp PCR fragment that was specific to C. falcatum. The DNA sequence of this fragment was determined and used to design oligonucleotides amplifying a 442 bp sequence characterized amplified region (SCAR). The specificity of the SCAR primers was evaluated using purified DNA from C. falcatum and other Colletotrichum spp. as templates in PCR. The results indicated that the SCAR primers were highly specific to C. falcatum since the 442 bp fragment was amplified only from DNA of isolates and races of C. falcatum but not from any other Colletotrichum spp. tested. The detection sensitivity of C. falcatum was 0.1 ng for genomic DNA of C. falcatum and 5 µg for DNA extracted from infected sugarcane
tissue. This new PCR-based assay is a convenient tool for detection of this important pathogen in seed canes to ensure production of sugarcane.

Materials and Methods

The ten different red rot isolates (Cf-06, Cf-1B, Cf-2B, Cf-Kushinagar, Cf-17, Cf-18, Cf-19, Cf-20, Cf-10 and Cf-01) were collected from 6 sugarcane varieties, CoS 8436, CoSe 95422, CoS 91269, CoJ 64, CoSe 92423 and CoLK 8102 which were purified and subcultured to the fresh Oat Meal Agar (OMA) medium.

DNA isolation and PCR amplification

Fungal DNA was extracted from 0.5g of fresh mycelial mat using a cetyl trimethyl ammonium bromide (CTAB) protocol (Saghai-Maroof et al., 1984), with a 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 known concentration of k-uncut DNA as standard. Equal amount of three such isolations were pooled and used as templates in the polymerase chain reaction (PCR).

PCR was carried out in 25 µl volume. A reaction tube contained 25 µg of DNA, 0.2 units of Taq DNA polymerase, 100 µM each of dNTPs, 1.5 mM MgCl2 and 5 pmol of decanucleotide primers. Amplification was performed by using universal primer pairs ITS-1 and ITS-4 using a DNA Engine thermal cycler (MJ Research, USA) following the protocol of Khanuja et al. (2000). Initial denaturation at 94 °C for 5 min, denaturation at 94°C for 10 sec, annealing at 60°C for 10 sec, extension at 72°C for 20 sec and final extension for 10 min at 72°C.

The amplified products of PCR were resolved on 1.2% (w/v) agarose gel containing 0.5 µg of ethidium bromide /ml using 1x TAE buffer. Resolved products were visualized and photographed under UV light source using the alpha-imager system.
Cloning and Sequencing
The amplified products on agarose gels were cut and eluted using GenElute Gel Extraction Kit (Sigma, USA) and the aliquot was quantified using a reference standard using MassRuler™ DNA ladder (MBI Fermentas, USA). The quantified PCR fragments were then ligated into pTZ57R/T vector and transformed into E. coli DH5α cells using bacterial transformation kit (Transformid, MBI Fermentas, USA). Positive recombinants were identified based on X-gal/IPTG selection. After screening the blue and white colonies, the white colonies were again checked for the presence of insert by colony –PCR. Positive colonies were used to isolate the plasmid using GenElute plasmid miniprep kit (Sigma, USA) and the inserts were released from the plasmid using restriction enzyme from the multiple cloning sites (MCS). Sequencing of plasmid DNA was carried out using ABI PRISM 377 DNA sequencer, using BigDye Terminator Cycle Sequencing. All nucleotide sequencing was performed at the DNA sequencing facility of 1st base, Selangor Darul Ehsan, Malaysia.

Sequence analysis and phylogenetic relationship
The nucleotide sequence data of three red rot isolates were analyzed using Genomatix DiAlign version 2 (Morgenstern, 1999) for sequence identities/similarities with the sequence data of other red rot isolates available in GenBank database. The sequences data of these isolates were analyzed for their molecular identification using the similar sequence data of selected red rot isolates reported from all over the world. The sequences were aligned using CLUSTAL W followed by MEGA 4.0 for the phylogenetic analysis (Tamura et al., 2007). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) were shown next to the branches and phylogenetic tree generated with Neighbour-Joining method and viewed by the NJ plot program.
Results

Detection of *C. falcatus* by PCR

The presence of *Colletotrichum falcatus* was demonstrated by electrophoresing the PCR products on agarose gels in all the ten isolates. Amplified product of the expected size (~550bp) was obtained from all the tested samples by using ITS-1 and ITS-4 universal primers specific to the fungus ITS gene. All the isolates, Cf-06, Cf-20, Cf-01, Cf-10, Cf-17, Cf-18, Cf-19, Cf-1B, Cf-2B, Cf-Kushinagar were found to be positive for *C. falcatus* infection from the collected source varieties (Plate 5.1). Therefore, infection of red rot was confirmed through the PCR analysis in all the collected varieties in the present investigation.

Plate 5.1 Agarose gel showing bands of ~ 550bp amplified by direct PCR with ITS-1/ITS-4 primers from red rot isolates (lanes: 1-10). 1 = Cf-06; 2 = Cf-20; 3 = Cf-01; 4 = Cf-1B; 5 = Cf-10; 6 = Cf-2B; 7 = Cf-Kushinagar; 8 = Cf-17; 9 = Cf-18; and 10 = Cf-19. Among the ten isolates (Lane 1-10), lane 1, 2, 5, 7, 8, 9 and 10 showing more prominent bands as compared to other lanes.

Sequence identity analysis and identification of red rot isolates

The ~550 bp amplicon with prominent intensity obtained from three important isolates (Cf-06, Cf-10, and Cf-17) of different varieties were cloned and sequenced. The analysis of the sequence data revealed 578 bp nucleotide. Consensus sequence data of three isolates (Cf-06, Cf-10, and Cf-17) were submitted in the Genbank with the accession numbers HQ833658, HQ833661 and HQ833662, respectively.

The NCBI BLASTn search analysis of red rot isolate Cf-06 (Acc. No. HQ833658) showed highest identities of 97-99 percent with *C. falcatus* isolates affecting sugarcane from Lucknow and Tamil Nadu from India (Table 5.2). However, sequence identities of 98-99 percent of Cf isolates-
10 and -17 under study were observed with *C. falcatum* isolates from Tamil Nadu and Lucknow, India (Table 5.2). Apart from Indian isolates, the present study isolates (Cf-06, Cf-10, and Cf-17) had a closer proximity with foreign isolates (90-91%) from Mexico (Acc. No. AF487427) and Japan (Acc. No. AB462376).

**Table: 5.2 Identity of present study isolates with other reported isolates from across the world**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Location</th>
<th>Isolate</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ833658</td>
<td>Shahjahnapur</td>
<td>Cf-06</td>
<td>97-99% (HM627380-T.N., HM592294-T.N., AY944745-T.N., AY944748-T.N., AB242422-L.K.O.); 90-91% (AF487427-Mexico., AB462376-Japan)</td>
</tr>
<tr>
<td>HQ833662</td>
<td>Karmha</td>
<td>Cf-17</td>
<td>98-99% (HM627380-T.N., HM592294-T.N., AY944747-T.N., AB242421-L.K.O., AB242412-L.K.O.); 90-91% (AF487427-Mexico, AB462376-Japan)</td>
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Nucleotide sequence similarity through Genomatrix dialing programme with Cf isolates (Cf-06, Cf-10 and Cf-17) showed highest
similarity 97-99% with *C. falcatus* isolates from India (HM627380, HM592294, AY944748, AB242422, AY944753, AB242421, AB242423, AB242412) (Table 5.3). The sequence similarity of three isolates (Cf-06, Cf-10 and Cf-17) confirmed that these isolates are highly similar to Indian isolate ‘*G. tucumanensis*’ rather than other countries isolate (Table 5.3).
Table 5.3: Percentage similarity (nucleotide) of three isolates of Sugarcane red rot with various isolates of *C. falcatum* based on Genomatix Dialign Programme

<table>
<thead>
<tr>
<th>isolate</th>
<th>% similarity</th>
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<tbody>
<tr>
<td>BQ898632_GKP_I ndia (675 bp)</td>
<td>0.977</td>
</tr>
<tr>
<td>AB242422_LKO_I ndia (605 bp)</td>
<td>0.961</td>
</tr>
<tr>
<td>HM627480_Tamil (577 bp)</td>
<td>0.931</td>
</tr>
<tr>
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<tr>
<td>HM627480_Tamil (577 bp)</td>
<td>0.931</td>
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Phylogenetic relationship of red rot isolates based on ITS region sequences

During the phylogenetic analysis of *C. falcum* sequences (HQ833658, HQ833662, and HQ833661), two clusters were formed along with the other selected *C. falcum* isolates. First cluster comprised of HQ833658 (Cf-06), HQ833662 (Cf-17) & HQ833661 (Cf-10) of India and these were found closely related with the *C. falcum* isolates reported from both Lucknow and Tamil Nadu regions of India (AB242423, AB242422, AB242421, AY944742, AY944753, AY944775, AY944748, AY944747, HM637380, HM592294). Isolate Cf-06 (HQ833658) made a separate sub-cluster with Cf-isolates from Tamil Nadu (AY944745) and Lucknow (AB242422) strains which showed its higher similarity. Isolate reported from Mexico (AF487427) and from Japan (AB462376) made separate cluster. The phylogenetic analysis of the three red rot isolates in the present study revealed that Cf-isolates HQ833661, HQ833658 & HQ833662 were closely related to red rot *C. falcum* isolates from India (Fig 5.1).

Based on highest sequence similarity of 97-99% and closest relationships at nucleotide level of three Cf-isolates in the present study with other reported Cf-sequences from India and abroad, the three studied Cf-isolates were confirmed as isolates of *Colletotrichum falcum*. 


Discussion

The ~550bp amplicon obtained from nested PCR was cloned and sequenced and the BLASTn search analysis revealed that *Colletotrichum falcatum* isolates identified in infected sugarcane in India was identical to isolates of the *Glomerella tucumanensis* reported from India.

According to the Suman *et al.* (2005), pathogen shows a great diversity in virulence as a number of pathotypes are known to occur in nature which have been classified on the basis of host differential reaction. As the host reaction is influenced by many climatic factors like temperature, humidity, time of inoculation, age of culture etc., the results sometimes become very confusing. Kumar *et al.* (2010) in his investigation showed that the high molecular diversity of the red rot isolates (Cf 01, 02, 03, 07, 08, and 09) through the RAPD, URP and ISSR markers from
differential commercials varieties grown in north India. Agnihotri (1990) suggested that the production of new strains in *C. falcatus* by hybridization could not be ruled out beside mutation. The disease is rudely spread in the susceptible varieties under favorable conditions. The rapid evolution of *C. falcatus* due to the plantation of susceptible varieties resulting in the emergence of new virulent strains causes breakdown in the varietal resistance (Mishra and Behera 2009). Variability in cultural and morphological characters and virulence, and development of physiological races, have been attributed to hybridization, mutation, conidial and hyphal fusions and heterokaryosis (Singh and Payak, 1968).

Nithya *et al.* (2012) developed a polymerase chain reaction (PCR) assay for accurate and sensitive detection of *C. falcatus* in planting materials. Randomly amplified polymorphic DNA (RAPD) analysis identified a 566 bp PCR fragment that was specific to *C. falcatus*. The DNA sequence of this fragment was determined and used to design oligonucleotides amplifying a 442 bp sequence characterized amplified region (SCAR). The specificity of the SCAR primers was evaluated using purified DNA from *C. falcatus* and other *Colletotrichum* spp. as templates in PCR. The results indicated that the SCAR primers were highly specific to *C. falcatus* since the 442 bp fragment was amplified only from DNA of isolates and races of *C. falcatus* but not from any other *Colletotrichum* spp. tested. The detection sensitivity of *C. falcatus* was 0.1 µg for genomic DNA of *C. falcatus* and 5 µg for DNA extracted from infected sugarcane tissue. This PCR-based assay is a convenient tool for detection of this important pathogen in seed canes to ensure production of disease free sugarcane. Ranjitham *et al.* (2012) also characterised 20 isolates of red rot pathogen from Tamil Nadu, India on the basis of morphology and PCR assays with ITS specific primers. Further confirmation was made with SCAR primers which amplified 442bp product indicating that all the isolates were *C. falcatus*. 
Based on highest sequence similarity of 97-99% and closest relationship at nucleotide level of three Cf-isolates in the present study with other reported Cf-sequences from India, the three studied Cf-isolates were confirmed as isolates of *Colletotrichum falcatum*.

Since the use of pathogen-free planting materials in commercial production is one of the most efficient ways to prevent this devastating disease, the PCR assay developed in this study may serve as a more sensitive means for the detection of *C. falcatum* and will consequently be helpful in the management of red rot disease of sugarcane.

**References**


