



UNIVERSITI PUTRA MALAYSIA

***GENETIC CHARACTERIZATION AND PATHOLOGICAL DIVERSITY OF
Colletotrichum falcatum Went ASSOCIATED WITH RED ROT DISEASE
OF SUGARCANE IN BANGLADESH***

MD. IMAM HOSSAIN

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SUGARCANE IN BANGLADESH**

By

MD. IMAM HOSSAIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

June 2022

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DEDICATION

This Thesis is dedicated to

*The most precious people in my life; my Beloved Parents
Late Md. Abul Hossain and Mst. AklimaAktar
My Lovely Wife Shahanara khatun
My Lovely son Rahat and daughter Sneha
My Beloved Brothers and sisters
For their unconditional everlasting love
They began my education
They motivated me to continue it
They will always contribute to it*



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**GENETIC CHARACTERIZATION AND PATHOLOGICAL DIVERSITY OF
Colletotrichum falcatum Went ASSOCIATED WITH RED ROT DISEASE OF
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June 2022

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Faculty : Agriculture

Red rot disease caused by *Colletotrichum falcatum* is the most destructive disease of sugarcane (*Saccharum officinarum* L.) in Bangladesh and worldwide. This disease causes major financial losses to the sugarcane growers as there is no effective method to control the disease in the field. Limited information is found on the characterization, virulence pattern and genetic variation of *C. falcatum*, associated with red rot disease in Bangladesh. Because of this, the objectives of this study were to characterize the *C. falcatum* isolates of sugarcane collected from different regions in Bangladesh based on morphological and molecular characteristics and to identify the virulence patterns based on the disease reaction in differential varieties, as well as to determine the genetic diversity of *C. falcatum* in Bangladesh using inter-simple sequence repeat (ISSR). From August 2018 to February 2019, a total of 41 *C. falcatum* isolates were obtained from sugarcane diseased samples collected from four regions in Bangladesh including Rajshahi, Rangpur, Khulna and Dhaka. The experiments were carried out at Bangladesh Sugarcrop Reserch Institute Pathology Laboratory and research farm using completely randomizd design (CRD) and randomized completely block design (RCBD) respectively. The molecular identification, genetic diversity and pathogenicity studies were carried out at the Bacteriology Laboratory, University Putra Malaysia. During the field survey, all sampling areas were infected by the red rot disease, and revealed that field disease incidence ranged from 3–25% in the study areas. All the isolates were characterized and identified based on morphological and molecular characteristics. The *C. falcatum* isolates showed diversity in morphological traits. Colony morphology varied from dark grey, whitish-grey, grey, and greyish-white colour, with flat, less fluffy, and raised fluffy mycelial topography, and the colonies had produced appressoria, seate and falcate shape, aseptate, and hyaline colour conidia. The four genes of the 41 isolates namely internal transcribed spacer (ITS-rDNA), β -tubulin, Actin, and glaceraldehydes -3 phosphate dehydrogenase (GAPDH) sequences showed a 99–100% similarity index to the conserved gene sequences in the GenBank database. The phylogenetic analysis of the four genes revealed that *C.*

falcatum isolates from Bangladesh were clustered in the same clade, and had no geographical distinctions. The four gene sequences also revealed that *C. falcatum* isolates from Bangladesh differed from other countries' isolates by nucleotide substitution at several loci positions. ISSR markers determined the low genetic diversity of the *C. falcatum* isolates in Bangladesh. Amongst the four populations of *C. falcatum*, population I (Rajshahi) had the highest genetic diversity level. While population II (Rangpur) had the lowest genetic diversity level. The cluster analysis divided the 41 isolates into four major clusters and each cluster was further divided into two sub-clusters. The clustering results revealed that *C. falcatum* isolates collected from the same location belong to the same sub-cluster, and a few of them belong to the other sub-clusters, indicating that the clusters were partially correlated to geographical regions and have a gene flow effect among the isolates. The virulence levels of the 41 *C. falcatum* isolates corresponding to disease reaction on the host plant were studied and were categorized into three main groups viz. virulent, moderately virulent, and least virulent. The seven sugarcane differential hosts were classified as resistant, moderately resistant, and susceptible. The sugarcane cultivars viz. Isd 18 and I-291-87 showed the highest level of susceptibility. While the cultivars Isd 39 and BSRI Akh45 showed the highest level of resistance. Finding reveal that *C. falcatum* isolates carried PKS1, HXT1, and Pel2 genes. The gene sequence analysis of the virulent, moderately virulent, and least virulent isolates revealed that the nucleotide substitution and deletion occurred at several loci. To the best of our knowledge, this is the first findings on the characterization, genetic diversity and virulence pattern of *C. falcatum* in Bangladesh. The results from this study could serve as a framework for establishing red rot disease control program in Bangladesh. Furthermore, this information is very crucial in developing red rot disease-resistant varieties for effective disease management strategies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**GENETIK PENCIRIAN SERTA DAN KEPELBAGAIAN PATOLOGIKAL
Colletotrichum falcatum Went DARIPADA PENYAKIT REPUT MERAH PADA
TANAMAN TEBU DI BANGLADESH**

Oleh

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Fakulti : Pertanian

Penyakit reput merah disebabkan oleh *Colletotrichum falcatum* adalah penyakit yang paling memudaratkan pada tanaman tebu (*Saccharum officinarum* L.) di Bangladesh dan dunia. Penyakit ini menyebabkan kerugian ekonomi yang besar kepada penanam tebu kerana tiada kaedah kawalan berkesan untuk mengawal penyakit tersebut di ladang. Maklumat berkenaan pencirian, corak virulensi dan variasi genetik *C. falcatum*, yang dikaitkan dengan penyakit reput merah di Bangladesh adalah sangat terhad. Oleh yang demikian, objektif kajian ini adalah untuk membuat pencirian penciran *C. falcatum* yang telah dipencilkan daripada kawasan penanaman tebu yang berbeza di Bangladesh berdasarkan ciri-ciri morfologi dan molekular; mengenalpasti corak virulensi berdasarkan tindak balas penyakit terhadap varieti yang berbeza; serta menentukan kepelbagaian genetik *C. falcatum* di Bangladesh menggunakan penanda molekular ulang jujukan antara mudah (ISSR). Dari Ogos 2018 hingga Februari 2019, sebanyak 41 penciran *C. falcatum* diperoleh daripada sampel tebu berpenyakit yang telah dikumpulkan daripada empat wilayah di Bangladesh iaitu Rajshahi, Rangpur, Khulna dan Dhaka. Eksperimen ini telah dijalankan di Makmal Patologi Institute Penyelidikan Tanaman Gula Bangladesh dan ladang penyelidikan menggunakan reka bentuk rawak sepenuhnya (CRD) dan reka bentuk blok secara rawak sepenuhnya (RCBD). Kajian pengenalpastian molekul, kepelbagaian genetik dan patogenik telah dijalankan di Makmal Bakteriologi, Universiti Putra Malaysia. Semasa tinjauan lapangan, semua kawasan persampelan telah dijangkiti oleh penyakit reput merah dengan kadar kejadian penyakit antara 3-25% di kawasan kajian. Semua penciran telah dicirikan dan dikenal pasti berdasarkan ciri morfologi dan molekular. Penciran *C. falcatum* menunjukkan kepelbagaian dalam ciri morfologi. Morfologi koloni berbeza-beza daripada kelabu gelap, kelabu keputihan, kelabu, dan warna putih kelabu, dengan topografi miselial yang rata, kurang gebu, timbul, dan koloni menghasilkan bentuk appresoria, mempunyai, *setae*, berbentuk bulan sabit aseptat dan konidia berwarna hialin. Empat gen daripada 41 penciran iaitu jujukan DNA-ribosom ruang trankripsi dalam (ITS-rDNA), β -tubulin, Actin, dan Dehid gliseral-3 fosfat dehidrogenase

(GAPDH) menunjukkan indeks persamaan 99-100% kepada jujukan gen di dalam pangkalan data GenBank. Analisis filogenetik bagi empat gen tersebut mendedahkan bahawa pencilan *C. falcatum* dari Bangladesh dikelompokkan dalam klad yang sama, dan tidak mempunyai pengaruh perbezaan geografi. Empat jujukan gen juga mendedahkan bahawa pencilan *C. falcatum* dari Bangladesh berbeza daripada pencilan negara lain melalui penggantian nukleotida pada beberapa kedudukan lokus. Penanda ISSR menunjukkan kepelbagaian genetik yang rendah bagi isolat *C. falcatum* di Bangladesh. Di antara empat populasi *C. falcatum*, populasi I (Rajshahi) mempunyai tahap kepelbagaian genetik yang paling tinggi. Manakala populasi II (Rangpur) mempunyai tahap kepelbagaian genetik yang paling rendah. Analisis kluster membahagikan 41 pencilan kepada empat kluster utama dan setiap kluster dibahagikan lagi kepada dua subkluster. Keputusan analisis kluster mendedahkan bahawa pencilan *C. falcatum* yang dikumpul dari lokasi yang sama tergolong dalam sub-kluster yang sama, dan beberapa daripadanya tergolong dalam sub-kluster yang lain, menunjukkan bahawa gugusan itu sebahagiannya dikaitkan dengan kesan geografi dan pengaruh aliran gen. kesan di kalangan pencilan. Tahap virulensi bagi 41 pencilan *C. falcatum* adalah berkadar dengan indak balas penyakit pada perumah telah dikaji dan dikategorikan kepada tiga kumpulan utama iaitu virulen, sederhana virulen, dan kurang virulen. Tujuh hos pembezaan dikelaskan sebagai sangat rintang, sederhana rintang dan rentan. Kultivar tebu iaitu. Isd 18 dan I-291-87 menunjukkan sangat rentan. Manakala kultivar Isd 39 dan BSRI Akh 45 menunjukkan sangat resisten. Dapatan mengesahkan *C. falcatum* mempunyai gen PKSI, HXTI dan Pel2. Analisis jujukan gen bagi isolat virulen, sederhana virulen dan kurang virulen mendapati terdapat penggantian dan pemadaman nukleotida di beberapa kedudukan lokus. Sepanjang pengetahuan kami, kajian ini adalah hasil penemuan pertama dalam pencirian, kepelbagaian genetik dan corak virulensi *C. falcatum* di Bangladesh. Dapatan kajian dapat dimanfaatkan untuk membina rangka kerja kawalan penyakit reput merah yang komprehensif di Bangladesh. Tambahan pula, maklumat ini sangat penting dalam membangunkan varieti tahan penyakit reput merah untuk strategi pengurusan penyakit berkesan.

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I certify that a Thesis Examination Committee has met on 10 June 2022 to conduct the final examination of Md. Imam Hossain on his thesis entitled "Genetic Characterization and Pathological Diversity of *Colletotrichum falcatum* Went Associated with Red Rot Disease of Sugarcane in Bangladesh" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
AMOVA	Analysis of Molecular Variation
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
<i>β-tubulin</i>	Beta tubulin
DNA	Deoxyribonucleic acid
DAI	Days after inoculation
ddH ₂ O	double distilled water
EDTA	Disodium ethylene diamine tetraacetate
FAO	Food and Agriculture Organization of the United Nations
GST	Mean coefficient of genetic diversity among populations
H	Nei's gene diversity
GAPDH	Glyceraldehydes-3 phosphate dehydrogenase
HXT1	Hexose transporter 1
Hs	Genetic diversity within populations
Ht	Total genetic diversity
I	Shannon's information index
ISSR	Inter-simple sequence repeat
ITS	Internal Transcribed Spacer
Kb	Kilo base
M	Molar
MEGA	Molecular Evolutionary Genetic Analysis

ML	Maximum Likelihood
mM	MilliMolar
MT	Millions Tones
Na	Average Observed number of alleles
Ne	Average effective number of alleles
Nm	Estimate of gene flow among populations
NAB	Number of Amplified Bands
NPB	Number of Polymorphic Bands
NMB	Number of Monomorphic Bands
NTSYS	Numerical Taxonomy and Multivariate Analysis System
pH	Potential Hydrogen
PPB	Percent of Polymorphic Bands
PDA	Potato Dextrose Agar
PCoA	Principal Co-ordinate Analysis
PKS1	Polyketide synthase
Pel2	Pectate lyase 2
NCBI	National Centre for Biotechnology Information
rRNA	Ribosomal Ribonucleic Acid
RAPD	Random Amplified Polymorphic DNA
RAPD	Random amplified polymorphic DNA
Rpm	Revolutions per minute
SAS	Statistical Analysis System
SSR	Simple sequence repeat
S.D	Standard deviation

Sec	Second
TBE	Tris borate EDTA
Tris	Tris (hydroxymethyl) amino methane
UPM	Universiti Putra Malaysia
UV	Ultra violet
UPGMA	Unweighted pairs group method of the arithmetic mean
V	Volts



CHAPTER 1

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an imperative industrial crop and the chief source of sugar in the sub-tropical and tropical regions worldwide (Patel et al., 2018). The worldwide sugarcane coverage was approximately 26.3 million hectares with a total production of 1907 million tonnes (FAOSTAT, 2019). In Bangladesh, sugarcane is ranked the second among the cash crops and occupies 0.11 million hectares of harvested land with a total production of 5.57 million metric tonnes (Rahman et al., 2016). Moreover, the sugar industry of Bangladesh is playing a significant contribution to improve the social and economic conditions of several people by creating job opportunities, providing a source of income, and developing rural infrastructure in the sugarcane growing area.

Sugarcane cultivation is challenged by a number of biotic and abiotic factors which are adversely affecting sugarcane productions (Talukdar et al., 2010). The abiotic causes are high temperature, dryness, extreme cold, tornados, floods, and other things (Sharma and Tamta, 2015). Amongst the biotic factors, the disease is the most important one. The lifecycle of the sugarcane plant is influenced by around 240 diseases (Rott et al., 2000). Approximately there are 10 viruses, 50 nematodes, 10 bacteria, and 100 fungi, have been identified as sugarcane pathogens around the world (Sharma and Tamta, 2015). In Bangladesh, a total of 40 sugarcane diseases have been reported. Out of them, 22 are caused by fungi, 4 by bacteria, 3 by viruses, and the rest are due to physiological and mechanical disorders (Talukder et al., 2010). Amongst the 40 diseases, red rot caused by *C. falcatum* Went (Perfect stage: *Glomerella tucumanensis* Speg.), which is also named *Physalospora tucumanensis* is the most devastating disease causing substantial reductions in sugarcane production in Bangladesh (Talukder et al., 2010). Red rot disease decreases sugarcane yield by 5–50% and sugar recovery by 31% (Ghazanfar and Kamran, 2016). Besides reducing the yield attributes, it also reduces the qualities of the juice (Thangamani et al., 2013).

The genus *Colletotrichum* has shown a wide variation in morphological characteristics when they were collected from different places (Prema et al., 2011). Accurate and quick detection of pathogens is important for the proper management of plant diseases (Singh et al., 2006). Primarily, *Colletotrichum* species have been described on the basis of morphological characteristics which are the basic conventional method for identification (Sutton et al., 1992, Arade et al., 2014). But the technique is highly influenced by environmental factors that affect the accurate identification of the pathogen (MacLean et al., 1993). On the other hand, molecular methods are faster, more accurate, and are usually not affected by environmental factors. For this reason, molecular approaches have become the best alternative approach for taxonomic studies and are effective methods for solving species identification problems (Cai et al., 2009). Recently, molecular methods such as sequence analysis of ITS-rDNA, β -tubulin, actin, and GAPDH have been utilized in the identification and phylogenetic analysis of many

Colletotrichum species (Nithya et al., 2012, Aktaruzzaman et al., 2018). Molecular phylogenetic studies assist to explain the contradictions in the conventional *Colletotrichum* classification system, and have provided fungal taxonomists with new powerful tools, including the ability for rapid detection of isolates and the elucidation of relationships between the fungal species (Shahnaji et al., 2013). Molecular techniques have also been utilized to differentiate within closely relevant species with less morphological variations and to distinguish strains within the species or even the particular isolates (Singh et al., 2006).

Several investigations have been conducted in controlling the economically important disease in sugarcane plantations. Cultural and chemical means such as removal of infected plants, stumblers, and setts treatment by using chemical fungicides are commonly used to control red rot disease. However, it may not be effective as the disease spread rapidly (Sharma et al., 2017). According to Guyot and Flori (2002), one of the most important control strategies for disease is to reduce the inoculum sources to prevent the disease from spreading further. In order to implement that strategy, Bayraktar et al., (2008) proposed that the knowledge of genetic diversity among the populations of pathogenic fungi would be beneficial in the enhancement of disease management systems and breeding purposes. A Similar suggestion was also emphasized by McDonald (1997) on the importance of studying the population biology for the fungal pathogen. Because, if there is high genetic diversity of the pathogen, the plant breeders give more attention to develop resistant variety and the pathologist could suggest using resistant variety to manage the red rot disease in the field condition. The inter simple sequence repeat (ISSR) marker has become an effective multilocus identifier in genetic diversity research and fingerprinting to better explain pathogen population dynamics and enable the development of successful management strategies (Bayraktar & Dolar, 2009, Arade, et al., 2014).

The assessment of pathogenic variations for plant pathogens prevailing in the crop-growing region is also the most important factor for successful management and improved crop production. Red rot disease could be managed only through the utilization of resistant varieties. But the *C. falcatum* changes its genetic make-up constantly through spontaneous natural mutation, heterokaryons, adaptation, and the pathogen shows enormous virulence diversity in nature that resulted in the evolution of new races (Kumar et al., 2011). These new isolates/pathotypes infects many popular sugarcane cultivars that are commercially cultivated in the farmer's field. Consequently, the popular sugarcane resistant cultivars became susceptible to the disease, and this led to the withdrawal of the cultivars from commercial cultivation. The assessment of virulence diversity enriches the knowledge of the current virulence patterns of *C. falcatum* and also helps in identifying the novel virulent isolates. This information is very important to plant pathologists and breeders to make a sustainable red rot disease management strategy for developing durable resistant varieties through the utilization of novel virulent isolates in varietal screening programs.

Colletotrichum falcatum is a well known pathogen that caused a severe red rot disease in the sugarcane. However, there is very little information on the morphology of *C. falcatum* isolates/pathotypes, their prevalence, and diversity (Kumar et al., 2011). The diversity of *C. falcatum* isolates has been documented by the use of morphological characteristics, host reactions, and the arbitrary markers method in other countries of the world (Suman et al., 2005). So, there is a vast scope to conduct a molecular study to identify and characterize the *C. falcatum* isolates and to group them according to their virulence and diversity in Bangladesh. In order to develop a resistant variety, plant breeders are needed to identify the virulent isolates/pathotypes of red rot disease. Sugarcane red rot disease has not been exclusively studied in Bangladesh. Accurate and quick pathogen detection is very important for proper disease management. There are several records of genetic characterization of other *Colletotrichum* species, but scanty evidence exists on *C. falcatum*'s distribution and diversity. For this reason, the current research was carried out with the following hypothesis and objectives:

Hypothesis

The hypothesis of this study is *Colletotrichum falcatum* associated with red rot disease on sugarcane is genetically diversified.

The main aim of this work is to characterize and elucidate the genetic and pathological diversity of red rot isolates in sugarcane growing areas of Bangladesh.

The specific objectives of this study are as follows:

- (1) To isolate, identify and characterize the *C. falcatum* associated with red rot disease in sugarcane-based on morphological and molecular techniques.
- (2) To determine the genetic diversity within the *C. falcatum* isolates by using ISSR markers.
- (3) To assess the virulence behaviour of the *C. falcatum*, and identify the pathogenicity related genes of *C. falcatum* isolates.

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