Protein profiling of *Colletotrichum falcatum*

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ABSTRACT

Red rot caused by *Colletotrichum falcatum* is a deleterious disease of sugarcane, responsible for 29.07 % loss in cane yield and 31 % loss in sugar production in Pakistan. Disease detection, at premature stage, will help to protect the crop from detrimental effects of red rot. Proteins extracted from *C. falcatum* can be used as an antigen source to design an immunological detection assay for the early detection of disease. Aim of present study was to identify *C. falcatum* and its protein profiling. Identification was done using morphological features and by amplification of Internal Transcribed Spacer (ITS) region. Total DNA was isolated from frozen mycelia by small-scale extraction method and PCR amplification was performed using primers (ITS1 and ITS4) that produced an amplification product of approximately 600bp. The purified PCR product was cloned and sequenced. Obtained DNA sequence showed the maximum sequence identity (99%) to *Gibberella moniliformis* (accession no. GU982311). Phylogenetic analysis also confirmed the similarity of this sequences with *Gibberella moniliformis.* Two types of conidia were observed under scanning electron micrograph in vicinity of mycelial culture, i.e. falcate shaped conidia as primary spores and oval shaped conidia as secondary spores. Total protein was extracted from frozen mycelia of *C. falcatum* through Trichloroacetic acid (TCA)/ acetone extraction method. Total protein was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) but a fainted band was observed. There may be two probable reasons for this: 1) due to less concentration of mycelial sample, which in turn resulted in a lesser amount of protein content, 2) *C. falcatum* has tough and sturdy cell wall which was unable to disrupt at room temperature.

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May the **ALMIGHTY ALLAH** bless all of you. Ameen!

Humdah Qayyum

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LIST OF ABBREVIATIONS

APS	Ammonium per sulfate
ASM	Acibenzolar S- methyl
bp	Base pair
BSA	Bovine serum albumin
CBB	Commassie brilliant blue
Cf	*Colletotrichum falcatum*
DAS-ELISA	Double antibody sandwich ELISA
DD-RT-PCR	Differential-display reverse transcription-PCR
DIBA	Dot immunobinding assay
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
GDP	Gross Domestic Product
ha	Hectare
HCL	Hydrochloric Acid
ITS	Internal transcribed spacer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo-base pair
kDa	Kilo Dalton
kg	Kilogram
LAMP	Loop mediated isothermal amplification

LM	Light microscope
MLM	Mixed linear model
min	Minutes
ml	Milliliter
mM	Millimolar
$Mgcl_2$	Magnesium chloride
ng	Nanogram
NGS	Next generation sequencing
Nacl	Sodium chloride
PAA	Polyacrylamide
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RAPD	Random Amplified Polymorphic DNA
RT-PCR	Reverse transcription PCR
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TLPs	Thaumatin like proteins
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

X-gal	5-Bromo-4-Chloro-3-Indolyl β-D galactopyranoside
μg	Microgram
μL	Microliter

Chapter 1

INTRODUCTION

1.1 Sugarcane

Sugarcane (*Saccharum officinarum* L.), a member of family *Poaceae* is a topmost gold mine crop cultivated widely in the world (Solomon, 2011) and used as a main source of sugar, bioethanol and bioelectricity production. The crop is responsible for ~70% white sugar needs and the remaining 30 % contributed by sugar beet. (Zurbier *et al*., 2008; Cardona, 2010; Sousa and Macedo, 2010).

Globally, sugarcane crop covers an area of about 20 million ha of 100 different countries with the approximate yield of 1325 million ha stalks per annum (Showler, 2016). Main sugarcane producing countries are Brazil, India, China, Thailand and Mexico. Pakistan ranks 4th in sugarcane production and 5th in sugar manufacturing around the world (Shahina *et al.,* 2007). According to second report of Pakistan provincial Government for 2016-2017, sugarcane covers an area of 1.225 million ha with an average productivity of 71.31 million tonnes (http://www.psmacentre.com/aboutus.php?id=6type=annual_review&status=1, last accessed on 1st July, 2017).

On basis of agriculture, sugar industry is the second largest industry of Pakistan with 86 sugar mills at different locations of the country that hires approximately 1.5 million people directly and indirectly. Almost 33 out of 86 are located in province Sindh whereas maximum 44 mills are positioned in province Punjab and remaining 9 in KPK province. Karachi Stock Exchange (KSE) has registered almost 43 % of sugar mills (http://jcrvis.com.pk/docs/Sugar201510.pdf, Last accessed on 25th July, 2017). Being the

second largest crop of Pakistan, sugarcane is contributing 0.6 % in the GDP of country (Pakistan economic survey, 2015-2016),

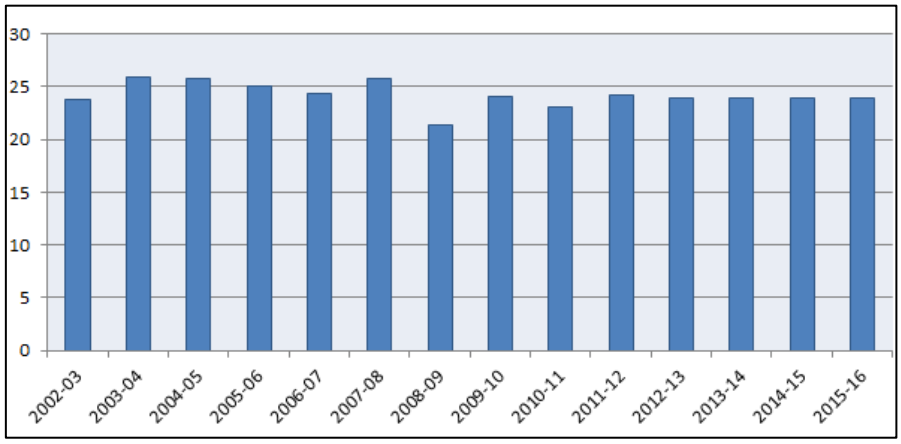

Figure 1.1: Sugar consumption per capita in Pakistan during 2002-2016 (**Source:** PSMA; Pakistan Sugar Mills Association**)**

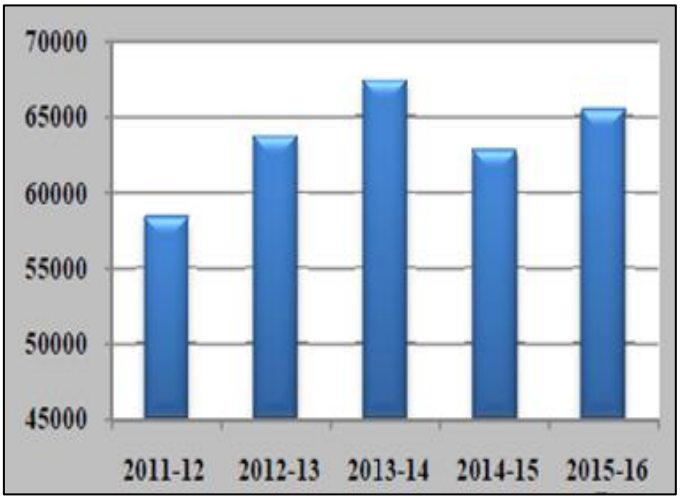

Figure 1.2: Sugarcane production during 2011-2016 (**Source**: PBS; Pakistan Bureau of Statistics)

Table 1.1: Survey of sugarcane production, area and yield produced during 1947-2015

(**Source**: Pakistan Sugar Mills Association)

Year	Area ('000 hectares)	Production ('000 tonnes)	Yield / Ha
1947-1950	202	6,775	33.54
1951-1955	245	7,193	29.36
1956-1960	366	10,319	28.19
1961-1965	469	15,849	33.79
1966-1970	582	22,312	38.34
1971-1975	608	21,647	35.6
1976-1980	757	27,994	36.98
1981-1985	897	33,580	37.44
1986-1990	823	32,656	39.68
1991-1995	927	40,902	44.12
1996-2000	1,030	48,371	46.96
2001-2005	1,009	48,343	47.91
2006-2010	1,063	54,365	51.1
2011-2012	1,016	56,740	55.8
2012-2013	1,128	63,719	56.5
2013-2014	1,172	67,428	57.55
2014-2015	1,150	63,203	54.96

In Pakistan, per capita appetite of sugarcane is 25 kg per annum (Bahadar *et al*., 2012). But its yield is quite low due to continuous damage of crop by several biotic and abiotic stresses (Long and Hensley,1972; Azevedo *et al*., 2011). Biotic stress factors that reduce the crop yield include weed growth, stalk borers injury caused by *Diatraea saccharalis* and *Eoreuma loftini* and pathogen infections including fungi, viruses and bacteria (Showler, 2013; Showler and Reagan, 2012; Souza *et al*., 2017). These pathogens are causing several devastating diseases on sugarcane plant. Different epidemics of sugarcane had reported due to increase in incidence of diseases (Souza *et al*., 2017).

Table 1.2: Pathogens infecting sugarcane/ diseases

(**Source**: Viswanathan and Rao, 2011)

Pathogen Type	**Species**	**Diseases**
Fungi	*Colletotrichum falcatum*	Red rot
	Fusarium moniliforme	Pokkah boeng
	Sporosorium scitamineuma	Smut
Viruses	*Sugarcane mosaic virus* *Sugarcane streak mosaic virus*	Mosaic
	Pea nut clump furovirus	Red leaf mottle
Bacteria	*Pseudomonas rubrilineans*	Red stripe
	Pseudomonas desaiana	Stinking rot

1.2 Red rot disease of sugarcane

Sugarcane is vulnerable to many fungal diseases. *C. falcatum* Went (perfect stage: *Glomerella tucumanensis* (Speg.) Arx andMuller), also called as *Physalospora tucumanensis* (Bailey and Jeger, 1992) is responsible for one of the most devastating disease of sugarcane

recognized as “red rot” (Viswanathan, 2010). Red rot was first described by Went in Java (Indonesia) in 1893 (Went, 1893) which was retitled as red rot by Butler in 1906 but in Pakistan, it was first reported in 1986 (Ahmed *et al.,* 1986). The Central and Northern areas of province Punjab (Pakistan) are mostly affected by the red rot (Khan *et al*., 2011).

Economic importance of this disease has revealed by many references as world has witnessed severe destructive outbreaks of red rot at different time periods (Ahmed *et al.*, 1986). Due to incessant evolution of new races of the pathogen, many important cultivars of sugarcane have been eliminating from the field (Malathi *et al*., 2010). Mainly sub-tropical countries are facing serious threat to sugar industry due to red rot (Alexander & Viswanathan, 2002; Viswanathan & Samiyappan, 2002).

C. falcatum produces an enzyme “invertase” that hydrolyzed the sucrose molecule (a disaccharide) specifically into its component monosaccharides named as glucose and fructose. Due to this hydrolyzation, amount of molasses increases in sugarcane and disease establishes (Sehtiya *et al*., 1993). That is why, it is also known as “Cancer” of sugarcane (Khan *et al*., 2011).

1.2.1 Taxonomic position of *Colletotrichum falcatum*

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Glomerellales

Family: Glomerellaceae

Genus: *Colletotrichum*

Species: *Colletotrichum falcatum*

(Maharachchikumbura *et al*., 2016)

1.2.2 Distinguishing features of red rot

When disease starts to appear in field, discoloration of leaves is considered as first symptom. During discoloration, all leaves of the crown start to wilt (Agnihotri, 1996). Then, minute reddish wounds appear on the upper surface of leaf along with some tiny red spots that develop on the upper surface of midrib in both directions. But problem starts when the most detrimental stage of disease happens by attacking the stalk (Duttamajumder, 2008). By splitting the stalk lengthwise, reddening of stalk tissues at right angle to long axis of stalk can be seen in internodes. Other distinctive symptoms include cross-wise white patches that may differ in number and size. Sometimes countless of white patches are present that gives the tissue a mottled impression (Viswanathan *et al*., 2011).

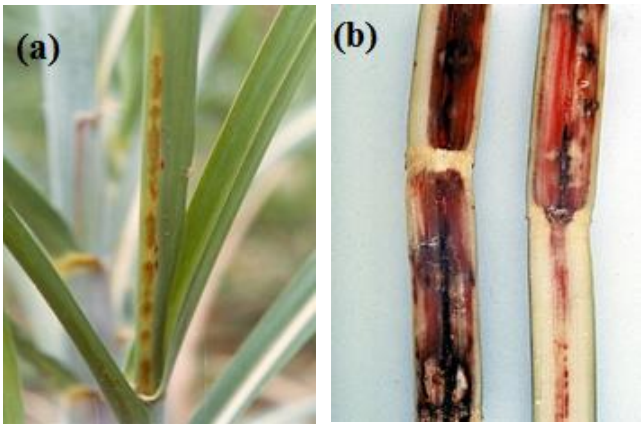

Figure 1.3: Symptoms of red rot disease. (a) Discoloration of leaves (b) Reddening of stalk tissues. (**Source**: http://istudy.pk/red-rot-of-sugarcane/, Last accessed on 1st July, 17)

1.2.3 Yield losses caused by red rot

Globally, red rot is responsible for 5-10 % per annum loss in sugarcane yield (Viswanathan and Samiyappan, 2002). In Pakistan, red rot is a major threat to sugarcane industry which is responsible for 29.07 % loss in cane weight and in result of this 31 % loss

in sugar recovery, 75% decrease of sucrose content and 90 % decrease in juice yield (Hussnain and Afghan, 2006). In addition to yield and quality losses, disease has been eliminating numerous vital sugarcane cultivars from the field and considered to be a biggest issue in many other sugarcane producing countries like USA, Bangladesh, India, Australia and Thailand (Viswanathan and Samiyappan, 2002).

1.2.4 Primary source of infection

Basically, *C. falcatum* is a sett borne pathogen, therefore, sett borne inoculum is considered as a primary source for disease development. In addition to setts, diseased debris, resting buds and infected planting material also take part in continuation of disease (Butler and Khan, 1913; Agnihotri *et al.*, 1979). But role of soil borne inoculum in spread of red rot is insignificant as *C. falcatum* cannot persist in the soil for more than 5-6 months (Chona and Nariani, 1954; Singh *et al*., 1977). *C. falcatum* infects the sugarcane plant during germination period by causing death of germinating seedlings (Agnihotri, 1996). Sometimes, post germination infections of new developing shoots occur due to presence of dormant mycelia in bud scales (Viswanathan *et al*., 2011).

Figure 1.4: Sets of sugarcane; primary source for red rot disease (**Source**: http://oobites.com/2012/06/bunch-of-sugar-canes/, Last accessed on, 1st July,17)

1.2.5 Secondary spread of disease

After primary spread of disease, secondary mode of transmission is also very important. During rainy season, irrigation and rain water are responsible for the dispersal of pathogen. But in winter, pathogen spreads by means of air streams (Agnihotri, 1990; Singh and Lal,1996). Conidia (asexually reproducing spores) primarily infects the stalk tissues through nodes. Due to sticky nature of conidial mass, spread of disease through air seems quite difficult. But presence of disease in upper portion of sugarcane stalks indicates the role of air in dispersal of conidia (Satyavir, 2003).

1.2.6 Mode of infection of *C. falcatum*

Pathogen enters the stalk through leaf scar, growth rings, root primordia and buds but nodes have a prime importance in causing the infection of sugarcane stalk (Steib and Chilton, 1951; Srinivasanan and Alexander, 1966; Singh *et al*., 1983). However, within the stalk pathogen spreads more rapidly through vascular bundles. (Abbot, 1938; Yin *et al*., 1996).

1.2.7 Identification of *C. falcatum*

Identification plays a significant role in field of taxonomy and it is an important aspect of it. By exact identification of an isolate, morphological and biochemical attributes of species can be identified, with vast and positive implications in different areas of research such as ecology and biodiversity (Hajibabaei *et al*., 2007; Seifert *et al*., 2007; Begerow *et al*., 2010). Generally, species are identified by following the taxonomic approaches, for example, classification and determination (Shenoy *et al*., 2007a). For specific recognition of a fungus, imperative characteristics like: morphology, physiology, specificity to the host and DNA sequence data are examined. In addition to this, relationship of specific fungus with closely

related extant fungi is perused that give rise to hierarchy of biological classification system (Summerbell *et al*., 2005; Shenoy *et al*., 2007a; Seifert, 2009).

Several significant attributes have been used for morphological identification of *C. falcatum*. It has both intercellular and intracellular mycelium and reproduces asexually by means of conidia formed on specialized spore producing hyphae known as conidiophores. Conidia of *C. falcatum* are usually colorless, thin walled, one celled, uninucleate and falcate shaped (also called as sickle shaped) and considered as a key morphological feature for its identification. For genetic characterization of species, telomorph's presence or absence, culture characteristics i.e., colony color and growth and pigments production plays a significant role. (Satyavir, 2003; Duttamajumder, 2008; Bailey and Jeger, 1992; Kumar *et al*., 2010).

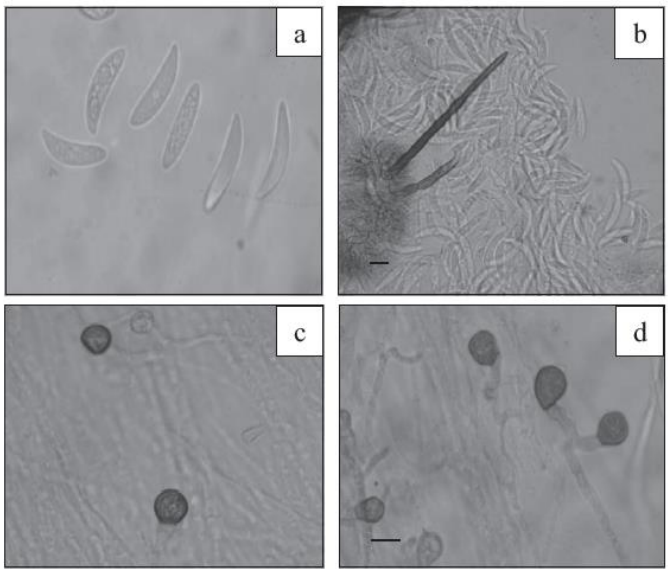

Figure 1.5: Morphological features of *C. falcatum*: (a) Conidia; (b) Setae; (c) Globose appresoria; (d) Clavate (Sangdit *et al*., 2014)

During past years, many *Colletotrichum* species have been characterized by molecular identification based on DNA via specific intergenic spacer region of ribosomal DNA repeats (Latha *et al*., 2003). Usually, fungal species are identified by means of ITS/ 5.8S rRNA gene

region (Soltis *et al*., 1999; Summerbell *et al*., 2005; Nilsson *et al*., 2009; Seifert 2009). Because with specific primer set, this region can easily be sequenced (White *et al*., 1990) by offering the proficient species-level resolution and it has competence to evolved quickly (Nilsson *et al*., 2008).

In recent times, ribosomal internal transcribed spacer (ITS) region has been asserted as a universal barcode marker for fungi (Schoch *et al*., 2012) and many pathotypes of *Colletotrichum falcatum* have characterized using this region (Malathi *et al*., 2010).

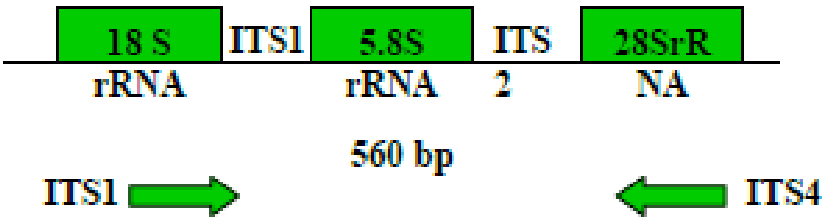

Figure 1.6: Structure of rRNA gene of genus *Colletotrichum* with specific ITS primers and their respective target regions (Kamle *et al*., 2013)

1.2.8 Detection of red rot disease

Red rot disease is impossible to diagnose in premature canes due to presence of dormant infections that remain unobserved and appeared after a period of 6-8 months (Viswanathan *et al*., 1998). Therefore, delicate, reliable and sensitive methods are needed for earlier detection of *Colletotrichum falcatum* in red rot infected canes. Traditional techniques such as inoculation tests by isolation and characterization of red rot pathogen is effortful and extensive.

Recently, use of immunological methods to tackle the red rot have received the attention of scientists (Schaad *et al*., 2003). Immunological techniques can be used for early detection and exact identification of pathogen on larger scale as well as these are easy, uncomplicated, swift, profitable, well defined and delicate (Narayanasamy, 2001 and 2005).

In immunological analysis, antibodies development is a most crucial step that includes vaccination of animals with highly purified proteins specific to the target organism (Harlow and Lane, 1988). And it is a big challenge to obtain satisfactory volume of protein from fungus mycelium (Gonzalez-Fernandez *et al*., 2014) due to presence of obstructive substances (Bianco and Perrotta, 2015). For differentiation and pre-fractionation of protein extracts, gel based analysis such as SDS-PAGE is a most widely used standard approach from a long period (Hernandez-Macedo *et al*., 2002).

1.3 Importance of present study

Sugarcane *(Saccharum officinarum* L.), is a predominant economical crop that is used as an energy source for human beings as well as a source of fuel for motor vehicles. It is cultivating in almost 100 countries around the world, covers an area of about 20 million ha and yielding 1325 million tonnes (Showler, 2016). Pakistan ranks 4th in sugarcane production and 5th in sugar production worldwide (Shahina *et al*., 2007).

Yield loss of sugarcane through infection of fungi, viruses, bacteria, phytoplasma and nematodes is a biggest problem. About 100 diverse diseases of sugarcane had reported due to attack of these pathogens (Bharti *et al*., 2012). In Pakistan, numerous diseases such as red rot, sugarcane mosaic virus, red and yellow stripes, pokkah boeng, whip smut and rust have been outlined (Anwar *et al*., 2010). Among these, red rot disease caused by *Colletotrichum falcatum* is responsible for 29.07 % loss in cane yield and 31 % loss in sugar production (Hussnain & Afghan, 2006).

Pakistan's economy relies on the sugar and sugarcane production as it is second largest cash crop contributing 0.6 % in GDP of the country (Pakistan economic survey, 2015-2016), But its production is comparatively low due to losses caused by red rot (Shahina *et al*., 2007).

Scientists are continuously striving to control the red rot disease by using different practices. Previously applied method to control it through use of disease free setts and application of fungicides were unsuccessful because fungicides molecular structure was easier to change to overcome resistance (Viswanathan and Samiyappan, 2000; Nallathambi *et al.*, 2000). Another method through development of disease resistant cane plant was the best one but due to outbreak of new lethal races and inconsistent behavior of *C. falcatum*, disease resistant varieties cannot survive more than 8-10 years (Yadav, 2006). One of the biggest challenge in control of disease is its detection at earlier developmental stages and it seems impossible due to appearance of characteristics symptoms at the later developmental stages. Therefore, suitable detection technique is needed to control the disease through production of diseased free setts (Schaad *et al.*, 2003).

Given the big success of immunological assay, present study is conducted for detection of red rot disease in immature canes, that will help to defend the sugarcane crop against destructive effects of red rot.

1.4 Aims and objectives

Following are the objectives of present study:

1. Protein extraction from *C. falcatum*

- Growth of *C. falcatum*
- Extraction of protein using TCA/ acetone extraction method.
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Chapter 2

REVIEW OF LITERATURE

Red rot disease induced by *C. falcatum* is considered as a highly devastating disease of sugarcane that causes a massive damage to the sugarcane crop across the world and is also responsible for the considerable economic loss by affecting sugar industry. So, there is a need to identify and detect this pathogen. Here, is a summary of work done on control of red rot disease in Pakistan and rest of the world.

Nayyar *et al.* (2017) developed the transgenic sugarcane plants resistant to red rot disease, caused by *C. falcatum*. *β-1,3-glucanase* gene isolated from *Trichoderma* specie was used to develop the red rot resistant sugarcane. In first generation plants (T0), transgene expression was measured by means of qRT-PCR and estimated expression was 4.4 times higher as compared to non-transgenics. Two pathogenic strains of *C. falcatum*; Cf08 and Cf09 were used for bioassay of transformed plants. Results of bioassay showed that some plants were totally resistant to Cf08 but resistance to Cf09 was relatively low. Electron microscopy of the sucrose filled stalk parenchyma cells of transgenic plants showed the lysis of hyphae of both strains of *C. falcatum* in the cells having adequate amount of sucrose in comparison to non-transgenic plants. Expression of the transgene was increased after infection of transgenic plants with *C. falcatum*. They found that active sites Glutamate 628 and Aspartate 569 of β-1,3-glucanase gene was responsible for the lysis and suppressive growth of hyphae as well as for cleavage of β-1,3-glycosidic bonds.

Scindiya *et al*. (2017) observed the variation in genes, responsible for virulence in *C. falcatum*, by using two phylogenetically different isolates namely Cf671 and Cf92020 that differ in the virulence behavior with 28 specific virulence gene homologs. Sequence analysis

of the genes viz, Pg1, PG2, HXT1, HXT3, PKS1, CreA, SCD1, RAC1, THR1, RGT2, Cap5, Cap20, ICL1, MAF1, ArpA, APH1, SSD1, Pel1 and Pel2 confirmed their pathogenic role and exposed both inter and intraspecific variation in genes. During host pathogen interaction, expression of the genes was also confirmed. This was a first study about the role of virulence genes and their expression in *C. falcatum*

Prittesh *et al.* (2016) isolated different strains of *C. falcatum* from the sugarcane producing areas of south Gujarat province, India. Nine strains were confirmed by amplification and sequencing of ITS rRNA gene. Pathogenic tests were conducted using nine sugarcane cultivars that revealed the resistant behavior of Co94004 cultivars against all strains of *C. falcatum*, however, cultivars Co671 were infected by all strains. Strain cfCHA showed the virulence frequency of 77.9 % by infecting maximum seven cultivars. Results of this study revealed the importance of Co94004 cultivar that was recommended to the farmers of that region to minimize the yield loss.

Rahul *et al*. (2016) described the mechanisms involved between *C. falcatum* (Cf) and sugarcane during interaction. Rather than field experiment, they developed an in vitro system using crude elicitor of *C. falcatum* and suspension cells of sugarcane to identify the genes involved in defense process as well as to analyze the transcriptome. *C. falcatum* elicitor was treated with sugarcane suspension cells of Co93009 cultivar and differential display RT-PCR was performed for transcriptome analysis after 3 hours and 30 minutes. Total 241 differentially expressed transcripts were obtained. 75 out of total 241 transcripts were used for cloning and sequencing analysis. About 37% of up-regulated transcripts were defense associated but the group of transcripts that were down-regulated had different criteria for grouping and contained approximately 27 % of differential transcripts. Many defense associated transcripts such as 14-3-3-like protein, calmodulin related protein, glycine rich

protein, Myb-related CBM2-like transcription factor, chitinase and antifungal peptide were discovered. For characterization of genes at domain level, RACE PCR was performed and complete genome of xylanase inhibitor and 14-3-3-like protein was determined. These results validated the usefulness of transcript profile in identifying the defense related genes in sugarcane against *C. falcatum*.

Singh *et al*. (2016) identified the marker-trait associations (MTAs) by genome wide mapping of 119 genotypes of sugarcane, imprinted for 944 SSR alleles, with aim to establish resistance against red rot disease caused by *C. falcatum*. Four marker-trait associations were identified with help of MLM (Mixed linear model) technique using TASSEL software that also contained kinship and population structure as co-factor. Around 10-16 % of trait variation was explained independently by these four MTAs. To identify the putative genes encoding defense related proteins, EST sequences of four MTAs were searched through NCBI blast onto genome sequence of sorghum. In proximity of these MTAs, many important genes named as Glycerol-3-phosphate transporter-1, Serine/ threonine-protein kinase, cytochrome P450, MAP Kinase-4 and Ring finger domain protein were confined. Prospected genes identified by this experiment proved to be of great importance in establishing resistance against red rot disease.

Viswanathan *et al*. (2016) analyzed genetic complexities in *C. falcatum* due to its nature to specifically attacks depository of sugarcane. They also identified peculiar behavior of *C. falcatum* by Next Generation Sequencing (NGS) technology and submitted the draft genome of pathogen that was approximately 48.16 Mb and contained 12,270 genes. Comparison of genome sequence obtained by next generation sequencing with other species of kingdom fungi had showed the association of *C. falcatum* with two other pathogens i.e. *C.*

graminicola and *C. sublineola*, responsible for anthracnose disease on maize and sorghum plants respectively.

Chandra *et al.* (2015) designed an assay for detection of *C. falcatum* and named it loop-mediated isothermal amplification (LAMP) assay. Total DNA was extracted from infected tissue and pure mycelia of fungus. Specific four primer sets were designed to amplify the exclusive DNA sequence of *C. falcatum*. Moreover, two closely related species of *C. falcatum* viz *C. fructivorum* and *C. acutatum* and a sugarcane infecting pathogen *Puccinia melanocephala* were used to test the specificity of this assay. But LAMP didn't show any reaction with these pathogens. Comparatively, with pure DNA of *C. falcatum*, LAMP showed ten times more specificity as compared to traditional PCR. They concluded that LAMP is a rapid and an easy method for detection of *C. falcatum* due to its specificity as well as its capability to examine the color change within an hour without additional post-amplification processing.

Sathyabhama *et al.* (2015) studied the molecular defense response established between sugarcane and *C. falcatum* right after interaction. As, visible symptoms of red rot disease appeared after 72 hours of pathogen inoculation therefore molecular events occur in that time were difficult to understand. To understand that process, they collected the sugarcane stalk samples from red rot resistant cultivar at 12 and 36 hours after inoculation and subjected to suppression subtractive hybridization. RNA was extracted from sugarcane variety "Co 93009" resistant to red rot and from susceptible variety "CoC 671" at 12 and 36 hours as well as from the mock samples of cultivar "Co 93009". During subtractive hybridization, RNA of susceptible and mock samples was used as driver whereas resistant variety's RNA was used as a tester for subtractions. At last, they collected total 139 EST's that were categorized by functions. Signal transduction and abundance of recognition EST's

were in high amount in twelve-hour response library but in 36-hour response library EST's corresponding to metabolism of nucleic acids were in high amount. Furthermore, real time PCR was performed to confirm the expression of gene in both resistant and susceptible varieties of sugarcane. That was the first report about primary defense responses after pathogen infection in red rot resistant cultivar.

Arade *et al*. (2014) characterized the different isolates of *C. falcatum* by observing their growth rate on PDA and by molecular identification. Isolates produced the colonies of different types and colors on PDA. Highest growth rate (87.34 mm) was observed by isolate SGCF-8, after incubating it for 10 days at 27±2ºC. Whereas maximum sporulation (24.67 million spores/ml) was observed by isolate SGCF- 5 and maximum dry mycelial weight was obtained in isolate SGCF-1 (184.67mg) by incubating it for 15 days. ISSR primers were used for molecular characterization and maximum polymorphism was depicted by primer UBC-873 (92.30%). Dendrogram results showed the collection of nine isolates in one group and sub group except one isolate (SGCF-4) that was located distinctly and showed 0.407 similarity matrix with SGCF-1.

Bharti *et al*. (2014) isolated the twenty-eight isolates of *C. falcatum* from red rot infected sugarcane plants. On PDA media plates and slants, these isolates exhibited totally different morphology. Fifteen isolates had produced the light color whereas thirteen produced the dark color colony. On basis of culture, five groups were formed such as dark non sporulating, dark sporulating, light non-sporulating, sporadically light sporulating and light sporulating. Measurements of conidia varied relative to each isolate but all of them had falcate shaped conidia. They also conducted a pathogenicity test to measure the variation within the virulence behavior of isolates. Six isolates "Cf908, Cf208, Cf508, Cf2109, Cf1608and Cf2609" out of twenty-eight were considered as more virulent. Results revealed

the variation within isolates of *C. falcatum* and also reported the six new races of pathogen in India.

Franco *et al.* (2014) identified the two homologues of BARWIN (Barley wound inducible protein) named as SUGARWIN1 and SUGARWIN2 in sugarcane and studied specific behavior of SUGARWIN2 against phytopathogens using *C. falcatum*. SUGARWIN2 were induced in sugarcane on damage caused by *Diatraea saccharalis* (a herbivore) instead of pathogen attack and also showed antimicrobial actions against *Fusarium verticillioides* that attacks sugarcane after *Dsaccharalis* attack. Role of SUGARWIN2 in cell death of *Aspergillus nidulans* (affect other plants except sugarcane) and yeast (*Saccharomyces cerevisiae*) was also verified. Recombinant SUGARWIN2 was responsible for the germline cell death by altering the morphology of *C. falcatum* through leakage of inner material, increase of vacuolization and fractures. But increase of vacuolization did not kill the *C. falcatum*. None of other fungus was affected by recombinant SUGARWIN2 indicating that SUGARWIN2 is specific to *C. falcatum*.

Sangdit *et al.* (2014) collected the fifteen isolates of *C. falcatum* from different locations of Thailand. For confirmation, PCR was performed using its1 and its4 primers unique to specific DNA fragment of 590 bp. Bands were sequenced and analyzed using GeneBank databases. Sequence analysis exhibited 95.32–100% identity of these isolates with each other and 96.30–97.74% with *C. falcatum*. Cultural characteristics of isolates were also different from each other. 5 isolates SBL1, KBL1-3, KBL1-1, KBL1-2 and KB1 produced grey colored colonies whereas remaining LB1-7, LB1-4, LB1-6, LB2-7, LB2-2, LB2-1, LB2-3, LB2-4, LB2-5, and NM1 had white color colonies. All isolates produced conidia, similar in shape and size to *C. falcatum* (approximately 2.38 to 4.76 μm thick and 21.42 to 28.56 μm lengthwise). Appresoria and setae were also produced by isolates. Furthermore, virulent

nature of these isolates was confirmed using plug method. Resistant cultivars of sugarcane K84-200 and K88-92 remained unaffected by each isolate of *C. falcatum* but susceptible cultivars E-Heaw and K93-236 showed high pathogenic rate and affected by almost nine isolates. Isolates that were collected from areas without red rot symptoms showed zero pathogenic behavior on both varieties. This study had differentiated the both pathogenic and non-pathogenic races of *C. falcatum* in Thailand based on their virulence nature.

Prathima *et al*. (2013) analyzed the defense response of sugarcane to *C. falcatum* (red rot causing agent). Fungus inoculated stalk samples were compared with control samples to find the difference. On a red rot resistant cultivar Co 93009, differential display RT PCR was carried out to observe the expression of genes after pathogen infection. About 300 differentially expressed transcripts were obtained by DD-RT-PCR. 112 out of 300 were selected for cloning and sequencing. Clones were further categorized into five groups. Defense and signaling response group showed that clones are homologous to the genes involved in defense mechanisms. They also observed the overexpression of some transcripts in this group that were related to plant defense process like jasmonic acid pathway and ethylene pathway. Demonstration of expression was performed using five imperative defense related genes. This was the first report about regulation of defense genes in sugarcane on pathogen attack.

Malathi and Viswanathan (2013) described the function of chitinase in control of red rot disease. They performed an experiment under both *in vivo* and *in vitro* conditions. Under *in vitro* conditions, chitinase production was noted by both bacterial and fungal antagonistic strains. It was observed that chitinase played a dominant role in suppressing the growth of pathogen. Bacterial strain (fluorescent pseudomonads) and fungal strains (*Trichoderma harzianum*) showed the positive association with chitinase under *in vivo*

conditions. A considerable increase in chitinase activity was noticed after *C. falcatum* inoculation. Role of sugarcane varieties in production of chitinase was also detected.

Bukhari *et al*. (2012) described a strategy to detect the *C. falcatum* in premature seed canes. After total protein extraction from mycelia of race Cf 05, two specific proteins of 27kDa and 45kDa were purified and used as a source of antigen to develop the polyclonal antibodies. New Zealand white rabbit were used for production of antibodies. Further, EISA and immunoblot were performed to test these antibodies against *C. falcatum*. Results of ELISA and immunoblot had confirmed the specificity and sensitivity of polyclonal antibodies against *C. falcatum*. Even dilution of 1:50,000 was quite enough to detect the pathogen. In western blot analysis, antisera developed against 27 kDa and 45 kDa protein bands, was found to react with these specific protein bands respectively. Results of this study reflected the specificity, sensitivity and suitability of antisera for detection of *C. falcatum*.

Malathi *et al*. (2012) identified the antifungal proteins against *C. falcatum*, causal agent of red rot disease. Various fungal and bacterial strains have antagonist's nature to suppress the growth of *C. falcatum* by production of chitinase and inactivation of metabolites. Therefore, antifungal proteins sources (toxin and suppressing enzymes) were identified by using these effective strains. Use of these antagonists against *C. falcatum*, inhibited the proteins production of this pathogen and inactivated the toxin and other metabolites. Results of this study confirmed the production of antifungal proteins by both fungal and bacterial strains. Further identification of these proteins was done by SDS.

Malathi and Viswanathan (2012) examined the variation in pathogenic effect of *C. falcatum* relative to host resistance in different sugarcane species. Twelve genotypes of sugarcane belonging to six different species viz., *Saccharum barberi*, *S. spontaneum*, *S. officinarum*, *S. sinense*, *S. robustum* and *Erianthus* were selected. Among all, *S. officinarum*

had high sucrose content as compared to other species. Results of cultural studies showed the negative correlation of virulence characters with resistance in host plant positive correlation with sucrose amount. These results also indicated the role of other factors in host resistance rather than sugar content. Different *C. falcatum* pathotypes exhibited the variation in selection of host plant. Less pathogenic strains of *C. falcatum* infected the sugarcane plants with reduced sucrose content. Results indicated that selection of sugarcane genotypes depend upon the virulence of pathogen.

Nithya *et al*. (2012) detected the *C. falcatum* using RAPD (also known as "Randomly amplified polymorphic DNA") analysis that produced a specific PCR band of 566 bp. After sequencing this fragment, primers were designed to amplify a region of 442 bp named as sequence characterized amplified region (SCAR). DNA was isolated from many species of genus *Colletotrichum* and specifically from *C. falcatum* to test the uniqueness of SCAR primers. Extracted DNA was used as template in PCR. Rather than amplification of DNA of other species, only DNA of *C. falcatum* was amplified by using SCAR primers. This indicated the detection sensitivity of 5 ng for DNA isolated from infected tissues of sugarcane and 0.1 ng for the DNA of *C. falcatum*.

Nithya *et al*. (2012) characterized the isolates of *C. falcatum* specie from the Tamil Nadu region of India based on genetic variability and toxin producing ability. Each isolate was found to produce the different concentration of toxin. Toxin produced by isolate Cf 671 caused the maximum electrolyte loss from leaves of sugarcane. Genetic relationship between these isolates was confirmed by RAPD analysis. Results of RAPD analysis exhibited the minimum and maximum similarity of 19 to 95 % between isolates of *C. falcatum*. Two clusters were recognized by UPGMA phylogenetic analysis. All isolates were sorted in

cluster B except one isolate Cf 98061 present in cluster A. However, in the dendrogram, no interaction was noticed between *C. falcatum* isolates and their ability for toxin production.

Malathi *et al.* (2011) collected the different isolates of *C. falcatum* from sugarcane cultivars growing in different regions of India and identified them by observing cultural and pathogenic behavior as well as through molecular analysis. For molecular analysis, 5.8s rDNA-ITS gene was amplified using specific ITS primers, Col1 and Col2. ITS sequence analysis sorted the Indian isolates into three separate clades named as Clade I, Clade II and Clade III. In Clade III, less virulent isolate similar to isolates present in other countries were found but among Clade I and II diversity of isolates was not encouraged by cultural and virulence behavior. This was attempted for the first time to perform the analysis on large collection of *C. falcatum* isolates using ITS based molecular approach and linked it to other traits as well.

Abbas *et al.* (2010) identified the morphological variations between four isolates of *C. falcatum*. These isolates were collected from four varieties of red rot infected sugarcanes viz, Co-1148, SHF-242, BF-162 and SPF-234. Isolates showed a great variation within the color range and in growth pattern. On basis of growth, two groups were formed. Isolates SPF-234 and SHF-242 produced thick colony while isolates BF-162 and Co-1148 had produced medium density colony. The colony color of cultivars SHF-242, CO-1148, SPF-234 and BF-162 ranged between red, orange, red bottom and brown respectively and showed that these isolates are morphologically very different. However, this study reflected a need to verify morphological variation among the isolates with respect to genetic diversity.

Prihastuti *et al.* (2010) isolated the *C. falcatum* from red rot infected sugarcane samples, collected from prototype zone. Type specimen of *C. falcatum* could not be discovered in any herbaria. Therefore, a neotype was assigned for *C. falcatum* to strengthen

the exertion of specimen name. By re-inoculation of pathogen in sugarcane, pathogenic nature of this ex-neotype was established again. Culture characteristics and morphology of conidia and appresoria was also examined. Results of phylogenetic analysis revealed the presence of *C. falcatum* group in in a different lineage of curved-spored *Colletotrichum* species.

Alvi *et al*. (2008) determined the genetic difference between sugarcane samples, resistant and susceptible to the red rot disease caused by *C. falcatum*. Five susceptible and twelve resistant genotypes were selected and RAPD (Random Amplified Polymorphic DNA) markers were used to find the difference at genetic level. A total of 300 markers and four genotypes were used initially for the experiment. Further 24 markers were selected from these 300 and tried on all genotypes. Polymorphic and monomorphic loci were differentiated from the total 182 loci produced. Number of monomorphic loci generated were 26 whereas polymorphic loci were 156 in number. Specifically, 29 monomorphic loci were generated for resistant genotype and 52 for susceptible genotype. But not a single locus might be exclusively associated with either susceptible or resistant behavior. Around 74.37% mean genetic similarity was recorded between the genotypes. This might be expected due to absence of parental diversity. Results showed that there was conceivably many genetic purposes behind resistance or susceptibility against red rot.

Sundar *et al*. (2006) demonstrated the role of synthetic signal inducers for inducing the resistance in sugarcane plants against *C. falcatum*. Various plant activators were used for inducing maximum resistance in sugarcane. And it was found that Acibenzolar S- methyl (ASM) constituted the considerable high amount of systemic acquired resistance (SAR) inside pathogen inoculated stalk tissues due to induction of peroxidase and polyphenyloxidases enzymes. ELISA was used to measure the pathogen concentration in

ASM treated tissues and it showed noticeable reduction of the pathogen spread in these tissues. Distinct cellular response was noticed in sugarcane suspension cultured cells by introduction of novel isoforms of polyphenyloxidases and peroxidases in *C. falcatum*.

Viswanathan *et al*. (2005) performed an experiment on sugarcane cultivars that vary in their resistance pattern for *C. falcatum* and studies the role of two pathogenesis-related proteins (PR) named as chitinases and thaumatin-like proteins (TLPs). By inoculation of cane tissues with pathogen, induction of these proteins was determined after specific time durations through western blot analysis. Four different chitinases proteins having molecular mass of 34, 35, 36 and 39 kDa were produced by resistant variety (Co 93009). These proteins became more intensified after six to forty-two hours of pathogen inoculation. Chitinase protein of 35 kDa was induced in the variety (CoC 671) prone to disease and a 26 kDa protein was confirmed in uninoculated tissues of each resistant and prone types. TLP proteins were induced in a similar pattern as that of chitinases protein. TLPs of 43 and 37.5 kDa were detected in higher amount in resistant sugarcane varieties whereas in variety prone to the disease such induction turned to be less extreme and might be seen eight and nine days after pathogen inoculation. The prevailing study suggests possible position of those pathogenic resistance proteins in producing red rot resistance.

Hiremath and Naik (2004) developed a protocol to detect the *C. falcatum* at early stages of red rot disease by using DIBA (Dot-immunobinding assay) procedure. Specific proteins used as an antigen source were separated using SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis). Different dilution of antisera of infected sample were prepared and nitrocellulose membrane was used to perform the DIBA. Dark blue precipitates were shown on the nitrocellulose membrane of DIBA due to reaction between antigen and

antibody. The appearance of these dark blue precipitates was an indication for the presence of disease on sugarcane

Viswanathan *et al*. (2003) examined the role of non-pathogenic rhizobacteria of *Pseudomonas* species for their capacity to combat the red rot disease by inducing systemic resistance in sugarcane against *C. falcatum*. Pathogen was inoculated on the sugarcane specifically at upper nodes and internodes of the stalk and native strains of *Pseudomonas* species were selected and applied at rhizosphere, a point distant from pathogen inoculation. In susceptible cultivar (CoC 671), less disease symptoms were observed after bacterial treatment. These bacterial strains lessened the effect of disease by producing pathogenesis related proteins. After Pseudomonas putida strain KKM 1 treatment, along with pathogen inoculation, a further increment in the action of pathogenesis related proteins (ß-l,3-glucanase and chitinase) was observed. In microtitre-plate assay, it was confirmed that chitinases hindered the growth of mycelia of *C. falcatum*. Western blot experiment uncovered the involvement of rhizobacteria in induction of new isoforms of chitinases as well as some new proteins such as thaumatin-like proteins and ß-l,3-glucanases. Results of this study indicated the antifungal activity of *Pseudomonas* strains in susceptible sugarcane plants by inducing the PR-proteins.

Viswanathan and Samiyappan (2002) evaluated the nature of bacterial strains, fluorescent *Pseudomonas* for inducing the systemic resistance in sugarcane plants infected with *C. falcatum*. In the field, these strains were sprinkled during planting of setts and then further applied two times using talc-based formulations. Sugarcane stalks treated with these bacterial strains were artificially inoculated with *C. falcatum* to measure the induced systemic resistant effect. In the sugarcane varieties, prone to the red rot disease, ISR response was outstandingly high as compared to other susceptible and resistant varieties. Different strains

of *Pseudomonas* were tested for their effectiveness against the red and three of them decreased the prevalence of disease. Fields treated with bacterial strains increased the germination of setts, crop production and sugar yield. This study suggests the role of bacterial strains in controlling the red rot disease.

Vohringer and Sander (2001) determined the relevance of antibodies produced in chicken egg yolk (IgY) by comparing with those produced in rabbits (IgG), based on detection mechanism. This comparison was made to detect the three strains of each fungal pathogens viz *C. falcatum* and *Fusarium subglutinans*. A model system was used to conduct this. Using indirect double antibody sandwitch (DAS) ELISA, fungus was detected along with all parameters quantified. Mycelia incubated for ten, twenty and fifty days on PDA at 24oC, was pooled and homogenized. Sample was quantified by measuring protein content. Sample with lowest fungus quantity (A045nm 0.1) was considered as the best for detection and antibody comparison. Results showed that IgY has equal accuracy as that of IgG and can be quantified in ten times large quantity as compared to IgG.

Chapter 3

MATERIALS AND METHODS

3.1 Sample collection

Fungal infected samples of sugarcane were collected from the local market of Islamabad, Pakistan.

3.2 Surface sterilization of samples

The infected tissues were cut into 4-5 mm pieces and surface sterilized by immersing in 1% sodium hypochlorite solution for one minute followed by washing with 70% ethanol. Then, pieces were washed three times with autoclaved distilled water and dried on sterilized tissue paper.

3.3 Fungal culture

Sterilized pieces were kept on PDA (Potato dextrose agar) media plates for 7 days at 25^{o}C in an incubator to grow the fungus. To obtain the pure colony of the fungus, growing edges of fungal colony developed from the infected tissue were picked and transferred it to another PDA plate. It was allowed to grow for 7 days at 25^{o}C. This procedure was repeated until the pure fungal colony was obtained and then, samples were stored at 4^{o}C.

3.4 Inoculum preparation

5 mm pieces of the PDA plate culture were cut out with a cutter and transferred to 500-ml flasks containing 150 ml potato dextrose broth (PDB). Then, samples were incubated in shaking incubator (130 rpm) on room temperature for 15 days to grow the mycelia.

3.5 Storage of mycelial sample

Grown mycelia was harvested after 15 days and separated from the PDB by filtration through cheesecloth. The mycelia were washed repeatedly with distilled water to remove the culture liquid and finally, stored at -80^{o}C until further processed.

3.6 Cultural and morphological identification

Cultural characteristics were observed by color and the texture of the colony grew on PDA plate. For morphological identification, slides were prepared by picking the fungal hyphae from the growing edges of the colony and examined under the light and scanning electron microscope to observe the conidia and mycelial characteristics.

3.7 Molecular identification

3.7.1 Extraction of genomic DNA

Genomic DNA was extracted from fungal mycelia for PCR analysis using small-scale method proposed by Coenen *et al,* (1997). 0.2g of frozen mycelia was ground into a fine powder using liquid nitrogen and transferred to a sterile 2ml centrifuge tube. Then, about 400 µL of extraction buffer (10 ml; 400µL of 20 mM EDTA [pH: 8.0], 200 µL of 20 mM Tris-HCl [pH 7.5], 1 mL of 1% SDS, 5mL 1% NaCl and 3.4mL sterile water) was mixed in the powder followed by brief vortex and incubated the homogenized mixture for 1 hour at 70^{o}C. After incubation, 400 µL of phenol and equal volume Sevag (chloroform and isoamyl alcohol [24:1]) were added into the slurry and centrifuged it at 11000 rpm (11500 xg) for 15 minutes. Centrifugation separated the mixture into two distinct layers. Upper aqueous layer was collected into a new microfuge tube and additional 550 µL sevag was added to the supernatant followed by centrifugation at 11000 rpm (11500 xg) for 10 minutes. Again, top layer was transferred to fresh tube and 100% ethanol was added in equivalent amount in it.

Sample was incubated at -20ºC for 4 hours for precipitation of DNA. Nucleic acids were pellet down by centrifugation for 20 min at 11000 rpm (11500 xg). A pellet was formed by precipitated DNA at the bottom of tube which was then washed with 70% ethanol for removal of impurities. Then, Pellet was air dried and dissolved in 40 µL sterile distilled water and stored at -20^{O}C.

3.7.2 Agarose gel electrophoresis

DNA was visualized using 1% agarose gel. To prepare the gel, 0.5 grams of agarose was added into 50 mL 1X TAE (0.5 mM EDTA [pH: 8.0] and 20 mM Tris Acetate) and heat it until solution became clear. 0.01% ethidium bromide was added into the precooled gel solution. Then, gel solution was poured into the suitable caster with required comb. When gel became solidified, load the DNA samples into the wells and run at 90 volts for 45 min. DNA was visualized under the UV light source.

3.7.3 PCR Amplification

PCR amplification of ITS region was done by using universal primers, ITS1-F (TCCGTAGGTGAACCTGCGC) and ITS4-R (TCCTCCGCTTATTGATATGC) (White *et al*., 1990). For amplification of targeted gene sequence, total 50 µL reaction contained 10X *Taq* buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 100 pM primers, 1 Unit of *Taq* polymerase and 2µL template DNA.

PCR reaction mixture was placed in thermal cycler which was set at following conditions: Pre-denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C/45 sec, primer annealing at 58 °C for 45 sec and extension at 72 °C/45 sec. However, Final extension was done at 72 °C for 7min. Then, amplified product was run on 1% agarose.

3.7.4 PCR product purification

GeneJET™ PCR purification kit (Fermentas; #K0701) was used to remove the primer dimers and to purify the PCR product. 45 µL of PCR product was mixed with the 45 µL binding buffer in a proportion of 1:1 and mixed thoroughly followed by brief vortex. Solution was transferred to GeneJet™ Purification column provided with the kit and centrifuged for 1 min at 12000 xg. Flow-through was discarded and about 700 µL of wash buffer was added into the column prior to centrifugation at 12000 xg for 1 minute. Flow-through was discarded again and empty column was centrifuged to completely remove the flow-through. Then, column was placed into a fresh 1.5 ml eppendorf tube and DNA was eluted into 50 µL elution buffer. Solution was centrifuged again for 1 min and supernatant was collected. Finally, purified PCR product was stored at -20^{o}C for further use.

3.7.5 Ligation of PCR product into pTZ57R/T vector

Ligation of PCR product with pTZ57R/T vector was done using Manufacturer's protocol (InsTAcloneTM PCR cloning kit, Fermentas). Total 30 µL ligation reaction was prepared that contained, 6 µL of 5X ligation buffer,10 µL of DNA, 3 µL of pTZ57R/T vector and 1 Unit of T4 DNA ligase (1 µL). Ligation mixture was kept overnight at 4^{o}C.

3.7.6 Transformation of DH5α competent cells

Electrocompetent cells of DH5α strain of *Escherichia coli* (*E. coli*) were transformed using "Electroporation method". To transform, 100 µL of ice thawed DH5α competent cells were taken in a 1.5 ml eppendorf and 15 µL of ligated product was added in it. Sample was mixed gently by flicking the tube and incubated for 10-15 minutes on ice. Then, incubated mixture was transferred to a pre-chilled electroporation cuvette (Gene Pulser®/Micropulser™). Cuvette was placed into electroporator and pulse of 1.4KV was

given. Cuvette was removed from the chamber immediately after electric shock and 1ml of LB (Luria Bertani) broth media (Sigma-Aldrich) was added into it. Mixture was transferred to a 1.5 ml eppendorf tube and incubated for 1 hour at 37ºC (100 rpm) to permit expression of antibiotic resistance gene. After incubation, cells were pellet down by centrifugation and pellet was dissolved in 100 µL of supernatant after discarding the rest of supernatant. At the end, cells were plated on LB agar plate supplemented with ampicillin, Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5- Bromo-4-Chloro-3-Indonyl-β-galactopyranosidase or X-gal and plates were incubated overnight at 37ºC.

3.7.7 Plasmid isolation from *Escherichia coli* (*E. coli*)

Single white colony from the plate was selected and added into 5ml LB broth supplemented with appropriate antibiotic. Sample was incubated at 37ºC (130 rpm) for 16 hours to promote the growth of bacteria. After 16 hours, media color was changed and became turbid due to rapid growth of bacterial cells. Culture was transferred into 1.5 ml eppendorf tube and centrifuged at 12000 xg for 15 min to pellet down the bacterial cells. Supernatant was discarded and plasmid was extracted from the pellet by using Gene Jet Plasmid Miniprep kit (Thermo ScientificTM; Cat # K0502). About 250 µL of re-suspension solution was added into the pellet and vortex it followed by addition of 250 µL lysis solution to lysed the bacterial cells by inverting the tube 4-6 times. After that, 350 µL of neutralization solution was added into the suspension and again invert it 4-6 times. Sample was centrifuged for 5 min at 12000 xg. Supernatant was transferred to Thermo Scientific GeneJET Spin Column and centrifuge for 1 minute. Flow through was discarded and about 500 µL of wash solution was added into the column and spin for 30-60 seconds at maximum speed. This washing step was repeated for two times to completely wash the pellet. Flow-through was discarded again and empty column was centrifuged for 1 minutes to remove the remaining flow-through. Then, column was transferred into a new tube and 50 µL elution buffer was

added into column followed by incubation for 2 minutes at room temperature and again centrifuge for 2 minutes at 12000 xg. Flow-through containing pure plasmid was collected in a new eppendorf and analyzed on 1 % agarose gel.

3.7.8 Glycerol stock preparation

To preserve the grown cultures of E. coli used for plasmid isolation, glycerol stocks were prepared. About 800 µL of grown culture was added into 200 µL of autoclaved 100 % glycerol and preserved at -80^{o}C until further processed.

3.7.9 Restriction digestion

Restriction digestion was performed to identify the plasmid by diagnostic digest. 1 U of enzyme was used in accordance with its suitable buffers for digesting 1 µg of DNA. Total 30 µL reaction was prepared for double digestion that contained 20 µL nuclease free water, 2 µL plasmid, 3 µL 10X Tango buffer, 1 µL of Eco-RI and 1 µL of Hind-III enzyme. For single digestion, 30 µL reaction contained 21 µL nuclease free water, 2 µL plasmid, 3 µL of 10X Tango buffer and 1 µL of enzyme. Then, mixture was incubated at 37^{o}C for 2 hours and analyzed on 1 % agarose gel.

3.7.10 DNA Sequencing

About 100ng/ µL of purified plasmid was sequenced using universal M13F [-20] primer from Macrogen, Korea. Obtained DNA sequence was compared with already submitted sequences in the GenBank databases through NCBI BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/).

3.7.11 Phylogenetic analysis

Almost 50 closely related sequences were retrieved from GenBank NCBI and a phylogenetic tree was constructed using Neighbor joining method of MEGA6 software with 1000 Bootstrap replicates.

3.8 Total protein extraction

3.8.1 Protein extraction from mycelia

Total protein was extracted from mycelial sample, using TCA/ acetone extraction protocol (Amalraj *et al.*, 2010).

In a precooled, autoclaved pestle and mortar, about 250 mg of frozen mycelia was grounded to fine powder using liquid nitrogen. The powder was then transferred to sterile 2 ml eppendorf and added 1ml of TCA/ acetone extraction buffer (10 ml; 1g TCA, 0.007g DTT and 10 ml acetone). To facilitate the precipitation, mixture was incubated for 1-hour at -20^{o}C and centrifugation was done at 12000 xg for 20min at room temperature using high speed mini centrifuge. After centrifugation, obtained pellet was washed with 1 ml of acetone wash buffer (10 ml; 0.007g DTT and 10 ml acetone) followed by brief vortex to homogenize the mixture. The homogenous mixture was placed again for 1-hour incubation at -20^{o}C and later centrifuged for 20 min at 12000 xg on room temperature. This washing step was repeated until the supernatant became clear. The final precipitated pellet was then crushed using liquid nitrogen until it became a dry powder and stored it at -80^{o}C (to remove the TCA from pellet). About 50 mg of powdered pellet was suspended in 100 ml LB and incubated at 37^{o}C for 1-hour followed by vortex after every 15 min. The sample was centrifuged at maximum speed and supernatant was collected. This centrifugation step was repeated to remove any contaminant and stored at -80^{o}C.

3.8.2 Protein extraction from plant leaves

Total protein was extracted from, the leaves of *Jasminum sambac* and *Morus alba* plants, as a control, using TCA/ acetone extraction protocol but temperature conditions were different from the protocol used for mycelial protein extraction.

About 250 mg of fresh leaf samples were crushed with liquid nitrogen in pestle and mortar. Then, 1 ml of TCA/ acetone extraction buffer was mixed into powdered sample taken in 2 ml eppendorf tube. Mixture was incubated for one hour at -20^{o}C and centrifuged at 4^{o}C for 20 minutes at 9,000 rpm. Obtained pellet was washed with 1ml of acetone wash buffer and vortex to completely dissolve the pellet in the wash buffer. The homogenous mixture was incubated again for one hour at -20^{o}C and centrifuged at 4^{o}C for 20 minutes at 9,000 rpm. By using liquid nitrogen, obtained precipitated pellet was crushed to fine powder and stored at -80^{o}C. Then, 50 mg of powdered pellet was dissolved in 100 ml of LB followed by incubation for one hour at 37^{o}C. During incubation, sample was vortex after every 15 minutes to dissolve the powdered pellet.

Then, it was centrifuged at 4^{o}C for 20 minutes at 9,000 rpm and supernatant was collected. This centrifugation step was performed one more time to remove the contaminants and finally stored at -80^{o}C.

3.8.3 Protein quantification

Bradford protein assay proposed by Bradford (1976) was used for the quantification of proteins. A standard was prepared using Bovine serum albumin (BSA) to obtained the linear regression equation. Different volumes of BSA solution 0 µL, 10 µL, 20 µL, 30 µL, 40 µL and 50 µL were taken in 1.5 ml eppendorf separately. Then, 1.5 ml Bradford reagent was added into each eppendorf and mixed gently. Mixture was incubated for 10 minutes at room

temperature and color change of solution was observed. Finally, absorbance was measured at 595 nm using a spectrophotometer. A standard curve was generated by taking volume (µL) of BSA on X-axis and absorbance on Y-axis. Absorbance of proteins extracted from leaves (control) and fungal mycelial samples was assessed using this process by taking the different volumes of unknown protein i.e. 10 µl, 20 µl, 30 µl, 40 µl and 50 µl.

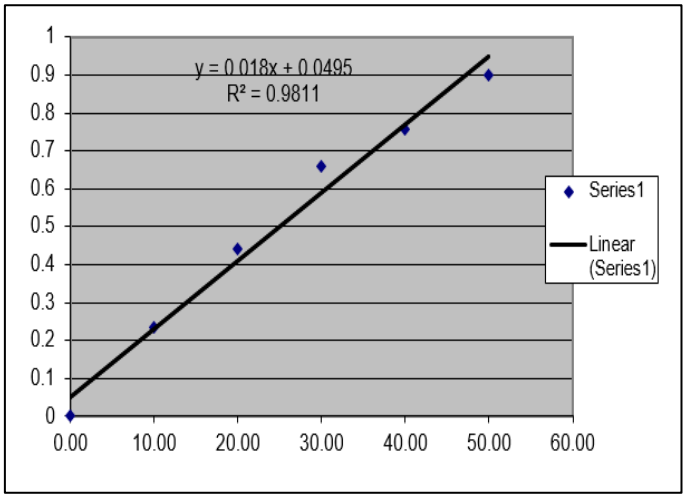

Figure 3.1: Standard curve generated by plotting BSA volume on X-axis and absorbance at Y-axis.

Then, amount of protein was calculated by means of absorbance value and linear regression equation. Following calculations were used for it:

µg of unknown protein (x) = Absorbance (y) - y intercept (b) / Slope (a)

Concentration assayed = µg of protein / µL assayed

Concentration of original solution = Concentration assayed × dilution factor

3.9 SDS PAGE

Protein samples were analyzed through Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to observe the quality of proteins.

3.9.1 SDS gel preparation

To prepare the SDS gel, reagents were mixed as given in the table 3.1.

Table 3.1: Reagents and their ratios used for SDS gel preparation

Reagents	**Resolving gel (12%)**	**Stacking gel (4%)**
Water	4.935 ml	4.48 ml
30 % PAA	6 ml	1 ml
Tris HCL (PH 8.8)	3.75 ml	-
Tris HCL (PH 6.8)	-	1.87 ml
10% SDS	150 µL	75 µL
10 % APS	150 µL	75 µL
TEMED	15 µL	7.5 µL
Total volume	**15 ml**	**7.5 ml**

APS (Ammonium per sulfate) and TEMED (Trimethylethylenediamine) were added just before pouring the gel. Glass plates were cleaned with ethanol and were assembled on casting stand along with spacers. Then, resolving gel was poured between the plates leaving about 2cm at the top. Water was added on the top surface of gel right after pouring the gel. When resolving gel was polymerized, water on the top of resolving gel was dried with a piece of tissue paper and stacking gel was added on the top of the resolving gel. Meanwhile, the comb was inserted in stacking gel and waited for 30 minutes for polymerization.

3.9.2 Sample preparation

For *Morus alba*, about 16.85 µL of protein sample quantified by Bradford assay was mixed with 13.15 µL of 2x laemmli buffer (10 ml; 5 ml of 0.5M Tris HCL (PH 6.8), 4 ml of 10 % SDS, 2 ml of glycerol, approximately 10 mg of bromophenolblue and 50 µL marceptoethanol was added in 950 µL aliquots prior to the gel loading) For *Jasminum sambac,*10.33 µL of protein sample was mixed with 19.67 µL of 2x laemmli buffer whereas for C. falcatum mycelial sample, 21 µL of protein and 9 µL of 2x laemmli buffer were mixed. Prepared samples were incubated on water bath for 3 minutes at 100^{o}C to denature the protein.

3.9.3 Sample loading

Before sample loading, wells were washed with a syringe to get rid of any unpolymerized polyacrylamide. After washing prepared samples were loaded in every lane and run in 1x SDS (5x stock; 15g Tris base, 144g glycine and 5g SDS) buffer at 40 volts for 45 minutes. When dye crossed the stacking gel and reached at resolving gel, then voltage was increased from 40 volts to 90 volts for 1 hour.

3.9.4 Staining and de-staining of SDS gel

After complete run, gel was placed in staining buffer (1L; 100 ml glacial acetic acid, 400 ml methanol, 500 ml distilled water and 1g Commassie Brilliant Blue [CBB]) for 1 hour to stain the protein bands. Then after 1 hour, gel was removed from staining solution (1L; 200 ml methanol, 100 ml glacial acetic acid and 700 ml distilled water) and de-stained by keeping it in de-staining solution for more than 24 hours. During de-staining of gel, de staining solution was changed continuously to obtain the appropriate results.

Chapter 4

RESULTS

4.1 Fungus isolation

Fungus isolated by inoculation of infected sugarcane pieces on PDA medium was identified by examining its cultural and morphological characteristics. White and fluffy mycelial colony was observed but from the bottom view, culture appeared to be light orange colored around periphery and gave some mauve shade radial patterns towards center (figure 4.1).

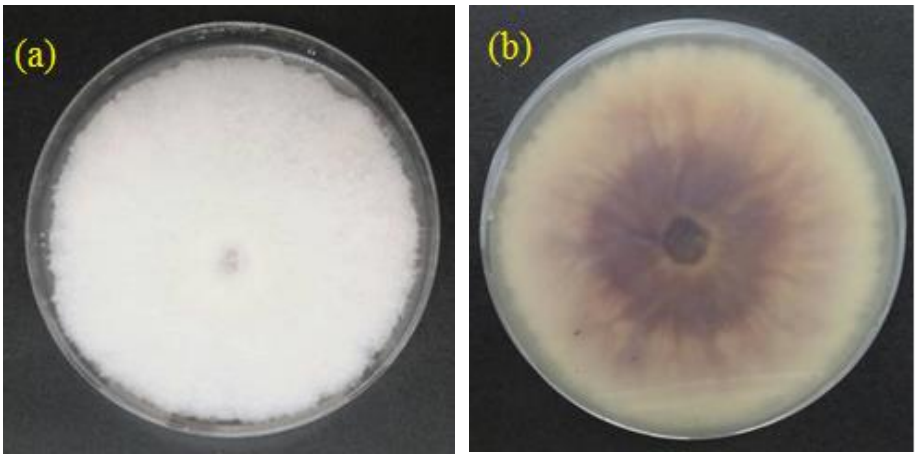

Figure 4.1: Pure fungal culture obtained through point inoculation. (a) Upper view (b) Bottom view

4.2 Identification based on morphological characteristics

Distinct morphological features of the isolated fungus were distinguished using light microscope (LM) and scanning electron microscope (SEM). Under light microscope, profusely branched and hyaline mycelia was observed along with falcate shaped conidia which is a unique character of *C. falcatum* (figure 4.2).

For further confirmation, mycelial culture and pattern of conidial formation were examined by scanning electron microscopy. Results of SEM showed the production of two types of spores by fungus. Falcate shaped conidia were produced as primary spores that formed the closely compact thick mycelium after germination (figure 4.3). Within this mycelial network, conidiogenic hypha were observed which produced oval shaped conidia as secondary spores. Mature oval conidia were also detected within the mycelial network as shown in figure 4.4.

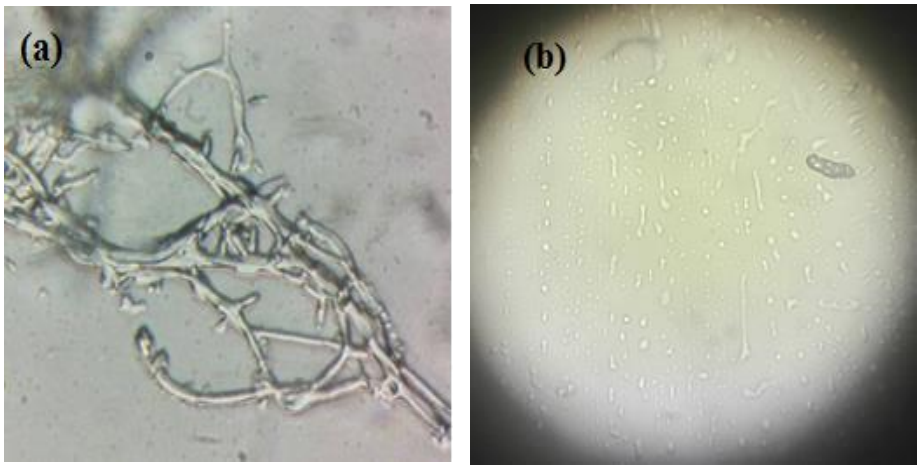

Figure 4.2: Morphology of *C. falcatum* using Light Microscope. (a) Mycelium of *C. falcatum.* (b) Falcate shaped conidia produced by *C. falcatum.*

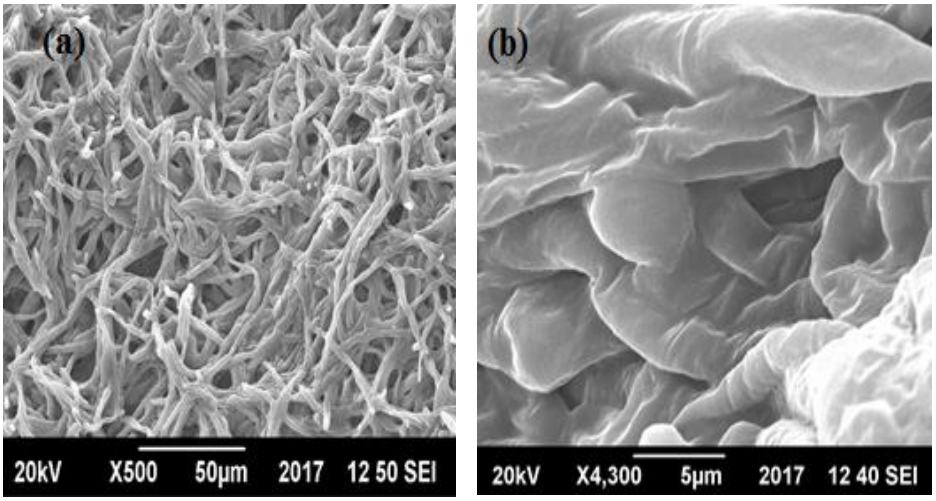

Figure 4.3: Morphology of *C. falcatum* using SEM. (a) Dense mycelia of *C. falcatum* formed from falcate conidia. (b) Mycelium of *C. falcatum*

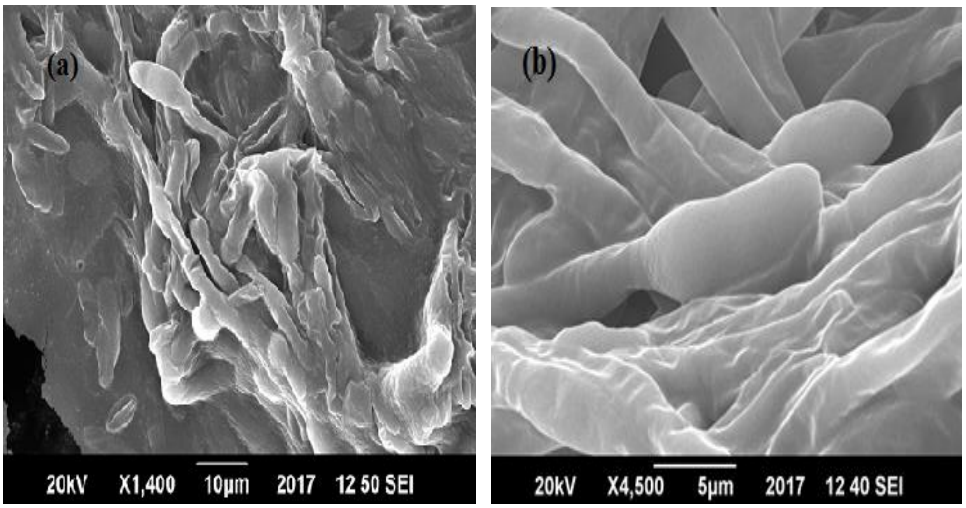

Figure 4.4: (a) and (b); Formation of oval shaped conidia at the tip of conidiogenic hypha

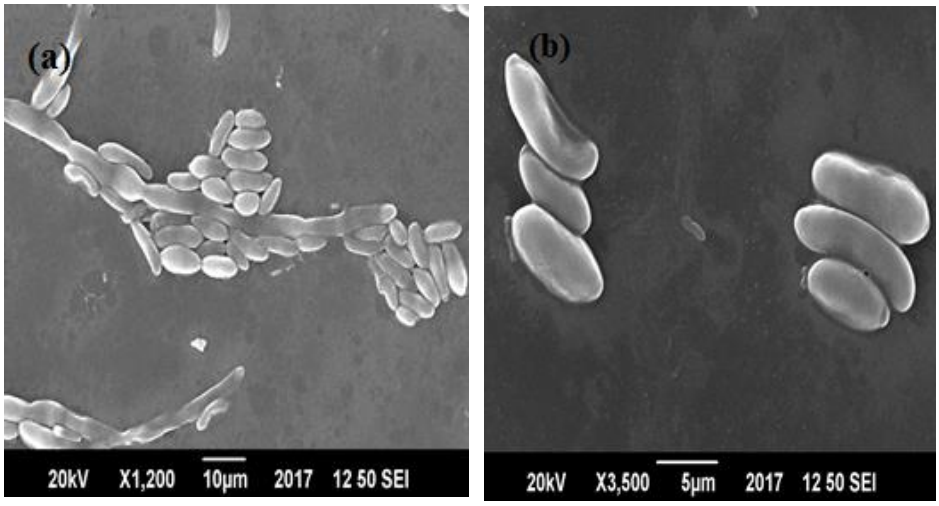

Figure 4.5: (a) and (b); Mature oval conidia after separation from conidiogenic hypha

4.3 Molecular identification of *C. falcatum*

4.3.1 DNA isolation

Total DNA was isolated from frozen mycelia of *C. falcatum* using small-scale extraction method and analyzed on 1 % agarose gel. Quality of isolated DNA was evaluated by comparison with 1 kb DNA marker. A good quality and clear DNA band was visible on gel as shown in figure 4.6.

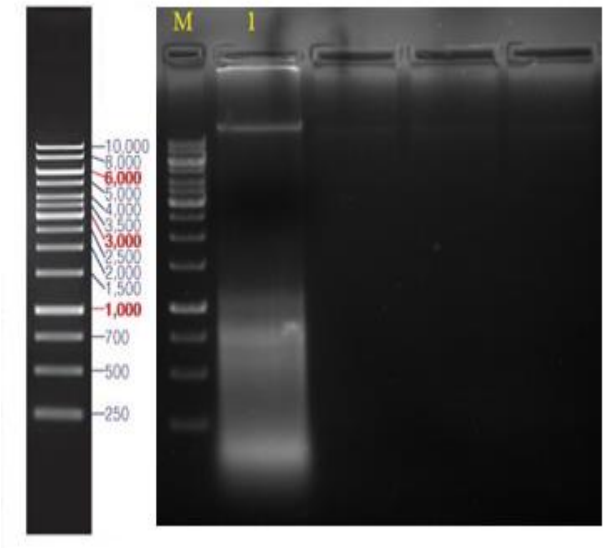

Figure 4.6: Agarose gel showing total DNA. Lane M: 1 kb marker and Lane 1: Total DNA isolated from fungal mycelia.

4.3.2 ITS based PCR amplification of DNA

ITS region (a part of nuclear ribosomal RNA cistron) was amplified using ITS1-F (forward) and ITS4-R (reverse) primers. Obtained PCR product was then analyzed on 1 % agarose gel along with 1 kb DNA marker to estimate the size of amplicon. Approximately, band of 600 bp (0.6 kb) was amplified from *C. falcatum* DNA as viewed in figure 4.7.

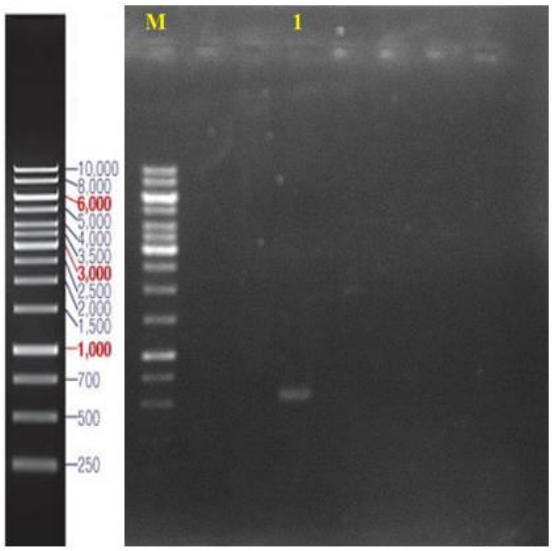

Figure 4.7: Agarose gel electrophoresis of ITS amplified PCR product. Lane M: 1 kb marker and Lane 1: ~0.6 kb ITS band.

4.3.3 Purification of PCR product

PCR product was purified using PCR purification kit (Thermoscientific). Purified product was then compared with standard 1kb marker to confirm the presence of purified gene band. The band of ~600 bp was observed at same position as that in PCR amplification (figure 4.8).

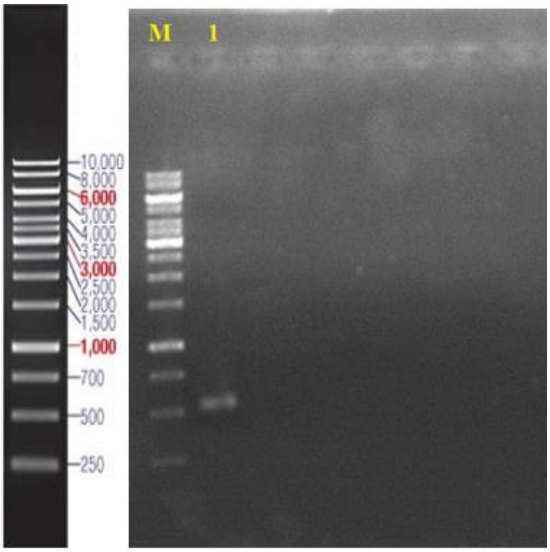

Figure 4.8: Agarose gel electrophoresis of purified PCR product. Lane M: 1 kb marker; Lane 1: ~0.6 kb ITS band.

4.3.4 T/A Cloning of *C. falcatum* DNA

Purified PCR product was then cloned in pTZ57R/T vector and transformation was done using electrocompetent DH5α cells of *E. coli*. After spreading of transformed culture on agar plate supplemented with suitable antibiotics, many blue and white colonies were appeared. Then, recombinant plasmid was isolated from the culture, obtained by inoculation of white colonies in LB broth.

4.3.5 Restriction digestion analysis

To confirm the successful cloning of insert, restriction digestion was performed on isolated plasmids using *EcoR*I and *Hind*III restriction enzymes. Size of digested product was examined on 1 % agarose gel. Figure 4.9 represents the digestion of plasmid by restriction enzymes in three different combinations. 1) By digesting the plasmid with *Hind*III enzyme, a single band of 3.5 kb corresponds to pTZ57R/T vector was obtained. This is due to presence of single restriction site of *Hind*III enzyme in vector, that linearized the DNA, and a single band was obtained. 2) Further, digestion of plasmid with only *EcoR*I enzyme, two bands of 3 kb and 0.3 kb were obtained. 3kb band corresponds to the pTZ57R/T vector band along with 0.3 kb insert band (instead of 0.6 kb). This reduction of band size is due to presence of additional *EcoR*I restriction site in the insert along with vector *3)* Moreover, the presence of *EcoR1* enzyme site in the insert was also confirmed by digesting the plasmid with *EcoR*I and *Hind*III restriction enzymes, same two bands of approximately 3 kb and 0.3 kb were obtained corresponding to vector and inserted gene.

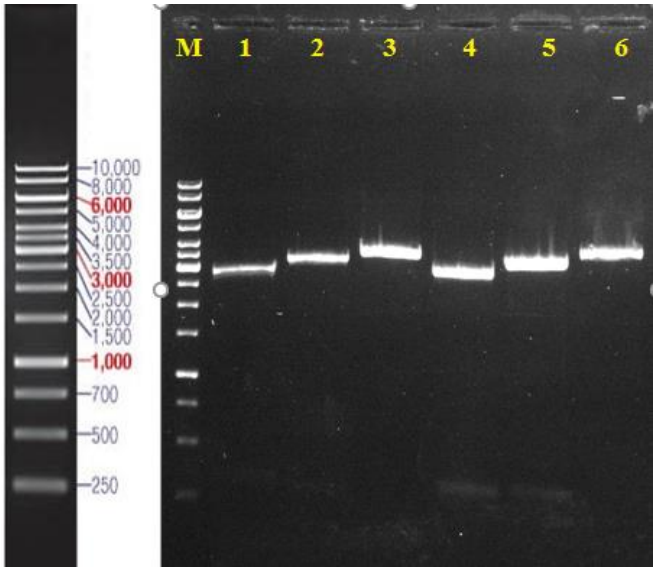

Figure 4.9: Restriction digestion of isolated plasmid with *Eco*RI and *Hind*III restriction enzymes. Lane M: 1 Kb marker; Lane 1 and 4: digested products of double digestion with *Eco*RI and *Hind*III (3 and 0.3 kb band); Lane 2 and 5: Digested products of single digestion with *Eco*RI (3 and 0.3 kb band); Lane 3 and 6: digested products of single digestion with *Hind*III (3.5 kb band).

4.3.6 DNA sequencing and analysis

Purified plasmids were sequenced using M13 forward (-20) primer. Obtained DNA sequence was BLAST searched into NCBI databases to determine its nucleotide sequence identity with already submitted sequences. Sequence exhibited the maximum sequence identity (99%) to *Gibberella moniliformis* (accession no. GU982311), also known as *Fusarium moniliforme, Fusarium verticillioides* and *Gibberella fujikuroi*.

4.3.7 Phylogenetic analysis

Almost 50 sequences were retrieved from GenBank NCBI based on high sequence identity in BLAST search. Sequences were aligned with Muscle and a phylogenetic tree was constructed using Neighbor joining method of MEGA6 software. 1000 Bootstrap replicates were applied

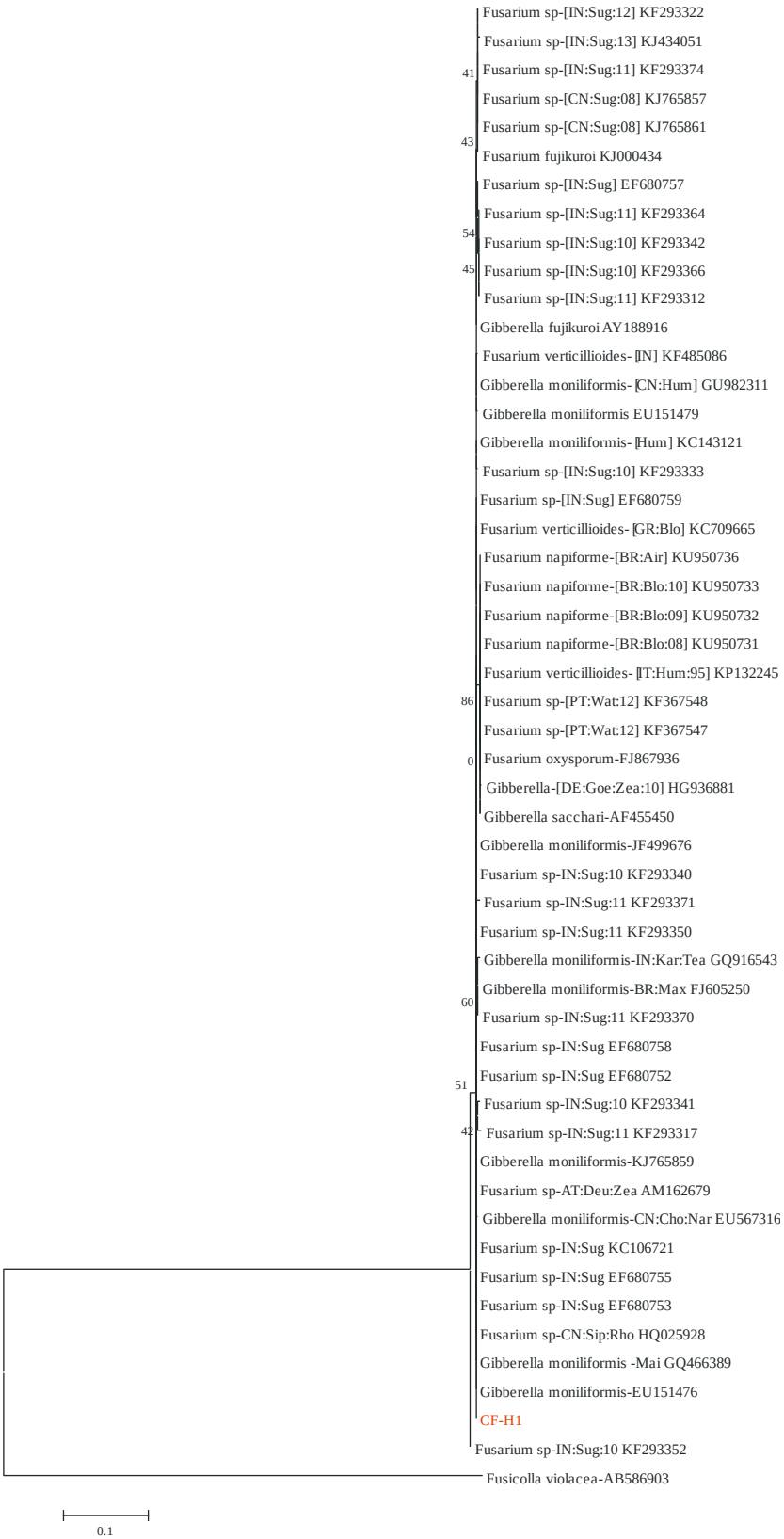

Figure 4.10: Neighbor joining tree (NJ) was constructed using MEGA6 software. Bootstrap (1000 replicates) values are written as percentages and scale bar at bottom shows nucleotide substitution value per site i.e. 0.1

for the reliability of phylogenetic tree. Each bootstrap value was written as percentage next to the branches of closely related taxa. Scale bar at base of tree described the nucleotide substitution value per site, i.e. 0.1. Phylogenetic analysis also confirmed the maximum similarity of present study sequence (CF-H1) with *Fusarium* species as shown in figure 4.10.

4.4 Protein extraction

Total protein was extracted using TCA/ acetone extraction protocol, from frozen mycelia of *C. falcatum* at room temperature. As a control, total protein was also extracted from the leaves of *Jasminum sambac* and *Morus alba* plants at 4^{o}C. Then, Bradford assay was performed to quantify the obtained proteins using bovine serum albumin (BSA) as a standard.

Results of Bradford assay described that protein sample extracted from *Jasminum sambac* and *Morus alba* leaves exhibited a slight color change at 10 µl, 20 µl, 30 µl and 40 µl dilutions. Maximum change in color from brown to blue was observed at 50 µl dilution with absorbance value of 0.17 and 0.123 respectively. But protein sample extracted from mycelia of *C. falcatum* did not show any color change at 10 µl, 20 µl, 30 µl and 40 µl. Although a very little change in color was noted at 50 µl dilution and absorbance measured for this dilution was 0.061. These results showed that a least amount of protein was present in mycelial sample. Then, obtained absorbance value was used to measure the volume of sample for SDS gel run and finally samples were run on 12 % SDS gel by comparing with page ruler protein ladder to observe the quality of protein.

Total protein extracted from mycelia, showed a faint band on SDS gel as shown in figure 4.11. However, protein samples extracted from leaves of *Jasminum sambac* and *Morus*

alba (control samples) showed a fair quality bands of 40 kDa and 55 kDa in each sample (figure 4.12).

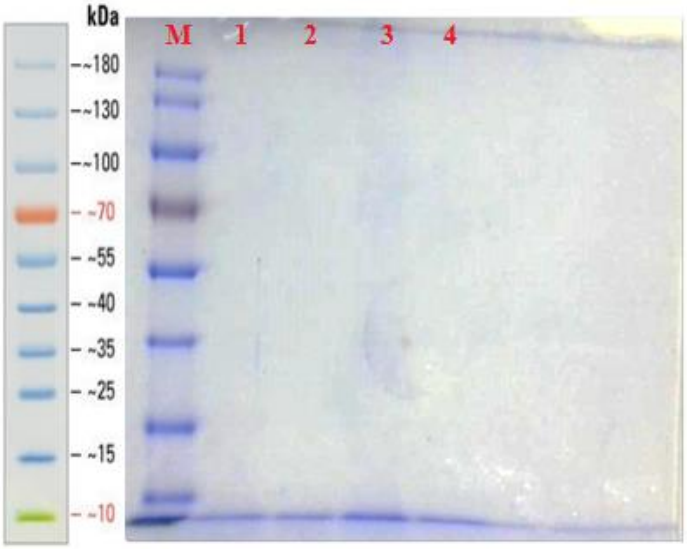

Figure 4.11: SDS-PAGE (12%) showing protein bands extracted from mycelia of *C. falcatum*. Lane M: Page ruler protein ladder; Lane 1, 2 and 4: No protein bands; Lane 3: Fainted band

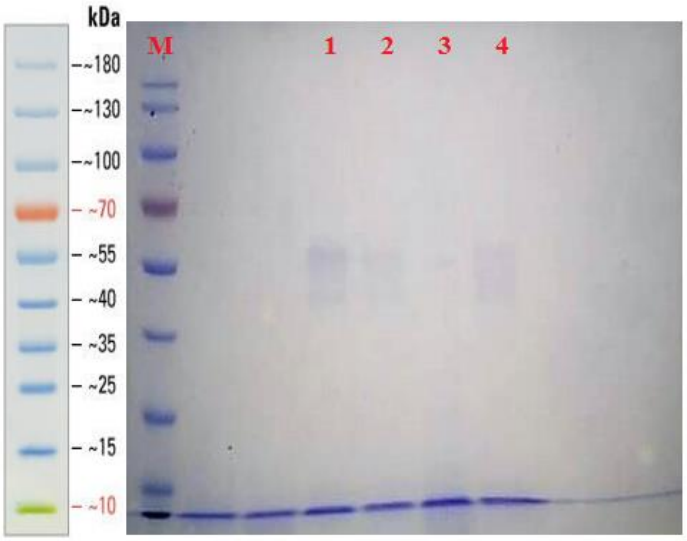

Figure 4.12: SDS-PAGE (12%) showing protein bands extracted from leaves of *Morus alba* and *Jasminum sambac* plants. Lane M: Page ruler protein ladder; Lane 1 and 2: protein bands of 55 and 40 kDa from leaves of *Morus alba*; Lane 3 and 4: protein bands of 55 and 40 kDa from leaves of *Jasminum sambac*

Chapter 5

DISCUSSION

Sugarcane (*Saccharum officinarum*) is one of the most extensively cultivated crop worldwide. Being the second largest crop of Pakistan, it is considered as backbone for the economy of country by satisfying 70 % white sugar requirement (Zurbier and Vooren, 2008; Cardona *et al*., 2010). It is also used as a major source of food, feed and fiber (Government of Pakistan, 2007). In Pakistan, sugarcane yield is quite low than average needs due to infections caused by pathogens like fungi, viruses and bacteria. About 15- 50 % losses of the sugarcane reported due to diseases caused by these pathogens (Martin and Wismer, 1989).

Among these pathogens, *C. falcatum*, causal agent of red rot disease of sugarcane, is considered as most destructive one. Almost 5-10% per year loss of sugarcane had reported due to red rot (Alexander & Viswanathan, 2002; Viswanathan & Samiyappan, 2002). Red rot affects the quality of sugarcane by depletion of sucrose content from the stalk tissues after pathogen infection (Sehtiya *et al.*, 1993). In addition to yield and quality losses, many vital sugarcane varieties have been eliminating due to red rot (Viswanathan & Samiyappan, 2002).

Control of disease through development of disease resistant varieties did not gave any successful results (Yadav, 2006) because at earlier stages of infection, disease cannot be detected (Schaad *et al.*, 2003) and planting of susceptible diseased sets causes breakdown of developed resistance by outbreak of new races of *C. falcatum* (Duttamajumdar, 2008). So, unavailability of suitable detection technique is a major factor in control of red rot disease (Viswanathan *et al.*, 1998; Hiremath & Naik, 2004). Risks of crop deterioration can be reduced at some level by detection of disease at earlier stages in seed canes (Schaad *et al.*, 2003).

In present study, *C. falcatum* was isolated and then identified by observing its morphological features and through amplification of ITS gene region. After that, its protein profiling was done for detection of red rot disease in premature seed canes.

After fungal isolation, mycelial culture was obtained that was white and fluffy in appearance. These obtained cultures were similar to the findings of Abbott (1938) who determined two kind of *C. falcatum* cultures i.e. one having dark greyish mycelia with compact and velvety texture while the other one was having cottony and white to light greyish mycelia.

Cultures were identified by observing morphological characteristics using light microscope and SEM. Under light microscope, falcate shaped conidia were observed along with profusely branched mycelial culture. Sangdit *et al*., (2014) reported the production of falcate shaped conidia by *C. falcatum.*

By SEM analysis, oval shaped conidia were observed along with falcate conidia in vicinity of germinating mycelium of *C. falcatum*. These oval shaped conidia were produced as secondary spores at the tip of conidiogenic hypha that was developed by germination of falcate conidia. Production of two types of conidia had already described by De Souza-Paccola *et al*., (2015) after infection of *Colletotrichum sublineolum* (a causal agent of anthracnose disease) on sorghum. Both falcate and oval shaped conidia were also detected in leaves of maize plant by infection of *C. graminicola* (Nishihara, 1975; Panaccione *et al*., 1989) which showed that formation of two types of conidia is a unique property of genus *Colletotrichum.*

Molecular identification of fungal culture was done by PCR amplification of 5.8S ITS region located between 18S and 28S rRNA genes because it is considered as best gene marker for fungal identification. Primers ITS1 and ITS4 were used for amplification of this

region and a band of approximately 600 bp was obtained. These primers were used by following the methodology of Sangdit *et al*., (2014) who identified the 15 isolates of *C. falcatum* by using ITS1 and ITS4 primers for amplification of 590 bp DNA fragment. This is also supported by the work of Malathi *et al*., (2010) who identified the significant pathotypes of *C. falcatum* by amplification of 5.8S ITS region.

Then cloning was done by ligation of amplicon in pTZ57R/T vector and positive clones were confirmed through restriction digestion analysis. For further confirmation, DNA sequencing of positive clones was performed using M13 forward primer. Obtained sequence showed the maximum sequence identity (99%) with *Gibberella moniliformis* (also known as *Fusarium moniliforme*). Lin *et al*., (2015) reported that in Malaysia, *Fusarium moniliforme* (causal agent of pokkah boeng disease of sugarcane) is known to cause red rot symptoms along with *C. falcatum* on sugarcane.

Total protein extraction from frozen mycelia of *C. falcatum* was performed using TCA/ acetone extraction protocol on room temperature. But a fainted band was obtained on 12 % SDS gel, due to less concentration of mycelial sample and due to robust and tough nature of fungal cell wall that is difficult to disrupt at room temperature.

Gonzalez-Fernandez *et al*., (2014) described that due to robust and vigorous nature of fungus, protein extraction from fungal mycelia is relatively problematic. This was also reported by Bowman and Free, (2006) who said that fungal species have very sturdy and tough cell wall that could cause hindrance during protein extraction. Osherov and May, (1998) stated that protein extraction from filamentous fungi is very tricky and exhausting due to presence of firm cell wall, ability of hyphae to form a clod and due to proteases action to degrade the protein rapidly.

In present study, as a control, proteins were also extracted from the *Jasminum sambac* and *Morus alba* plant leaves using TCA/ acetone extraction protocol at 4^{o}C to evaluate the effectiveness of the protocol and effect of temperature on extraction. Remarkable bands of 55kDa and 40 kDa were found on 12 % SDS gel.

Role of temperature in protein extraction was described by Cseke *et al*., (2004) they said that proteins should be extracted at tremendously low temperature (4^{o}C) due to their ability to denature after releasing from cell through cell lysis.

It has already been described that by using TCA/ acetone protocol, proteins are difficult to extract from complex tissues such as mycelia but on the young plant samples, it proved to be very effective (Chatter jee, 2012; Carpentier *et al*., 2005; Saravanan and Rose, 2004; Wang *et al*., 2003). But after precipitation, resolubilization of proteins is very complicated with TCA/ acetone extraction protocol (Carpentier *et al*., 2005).

CONCLUSION

Proteins extracted from mycelia of *C. falcatum* through TCA/ acetone extraction method produced a fainted band on SDS PAGE due to less concentration of mycelial sample and due to presence of tough and robust cell wall of mycelia which was unable to disrupt at room temperature. Therefore, optimization of this protocol is required to obtain the adequate amount of protein from mycelial sample. In this study, obtained DNA sequence showed the maximum sequence identity (99%) to *Gibberella moniliformis* (named as *Fusarium moniliforme*) that is also known to cause red rot symptoms in sugarcane along with *C. falcatum.*

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APPENDICES

Appendix A

MEDIA COMPOSITION FOR FUNGUS CULTURE

Potato dextrose agar (PDA) media	**Amount per Liter**
Potato infusion	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1 Liter

Potato dextrose broth (PDB) media	**Amount per Liter**
Potato infusion	200 g
Dextrose	20 g
Distilled water	1 Liter

Appendix B

MEDIA COMPOSITION FOR BACTERIAL CULTURE

Nutrient agar media	**Amount per Liter**
Peptone	5g
Sodium chloride	5g
Beef extract	1.5g
Yeast extract	1.5g
Agar	15g
Water	1 Liter

Luria Bertani (LB) Broth media	**Amount per Liter**
Tryptone	10g
Yeast extract	5g
Sodium chloride	10g
Water	1 Liter