GENETIC DIVERSITY, PATHOGENICITY AND SECONDARY METABOLITES OF *Fusarium* spp. ASSOCIATED WITH BAKANAE DISEASE OF RICE IN MALAYSIA AND BANGLADESH

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
bp	base pair
^{o}C	degree Celsius
cm	Centimetre
hr	Hour
g	Gram
kb	kilo base
L	Litre
M	Molar
min	Minutes
mA	micro ampere
µl	Microlitre
ml	Millilitre
mm	Millimetre
n	total number
rpm	rate per minute
v	Volt
$\chi 2$	Goodness of fit test

LIST OF ABBREVIATIONS

AMOVA	Analysis of Molecular Variance
AV	Avirulence
BLAST	Basic Local Alignment Search Tool
CA	Carrot agar
CLA	Carnation Leaf-pieces Agar
CM	Complete medium
$CuSO_4$	Cuprum sulphate
dai	Days after inoculation
dH_2O	Deionized distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSI	Disease severity index
F.	*Fusarium*
EtBr	Ethidium Bromide
FA	Fusaric acid
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
FFSC	*Fusarium fujikuroi* species complex
FGSC	*Fusarium graminearum* species complex
FIESC	*Fusarium incarnatum-equiseti* species complex
FOSC	*Fusarium oxysporum* species complex
FSAMSC	*Fusarium sambucinum* species complex
FSSC	*Fusarium solani* species complex
GA_3	Gibberellic acid
GenAlEx	Genetic Analysis in Excel
GFSC	*Gibberella fujikuroi* species complex
HCl	Hydrochloride acid
H_3BO_3	Boric acid
HV	Highly virulence
KCl	Potassium chloride
KH_2PO_4	Potassium hydrogen phosphate
MCMC	Markov Chain Monte Carlo

MEGA	Molecular Evolutionary Genetic Analysis
MeOH	Methanol
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulphate
ML	Maximum likelihood
MLST	Multilocus Sequence Typing
MLSA	Multilocus Sequence Analysis
MoO_3	Molybdenum oxide
MON	Moniliformin
MV	Moderately virulence
NaCl	Natrium chloride
Na_2HPO_4	Sodium hydrogen phosphate
NaOCl	Sodium hypochlorite
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Broth Agar
PPA	Peptone Pentachloronitrobenzene Agar
RBD	Rice Bakanae Disease
RPB2	RNA polymerase II subunit 2
SA	Soil agar
SNA	Spezieller Nährstoffarmer Agar
SPSS	Statistical Package for Social Science
spp.	Species
SSR	Simple Sequence Repeats
TBE	Tris Borate-EDTA
TEF-1α	Translation Elongation Factor1-α
UPLC	Ultra Performance Liquid Chromatography
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Virulence
WA	Water agar

LIST OF APPENDICES

KEPELBAGAIAN GENETIK, KEPATOGENAN DAN METABOLIT SEKUNDER *Fusarium* spp. YANG BERKAIT DENGAN PENYAKIT BAKANAE PADA PADI DI MALAYSIA DAN BANGLADESH

ABSTRAK

Penyakit bakanae merupakan satu penyakit yang sedang memuncul pada padi dan ia menjadi masalah yang penting di negara-negara penanaman padi di Asia termasuk Bangladesh dan Malaysia. Penyakit ini boleh mengehadkan pengeluaran beras jatuh ke paras 30%. *Fusarium fujikuroi* diketahui sebagai penyebab kepada penyakit bakanae manakala terdapat beberapa spesies lain yang berkait dengan penyakit ini. Populasi spesies *Fusarium* pada masa kini mungkin berbeza dengan laporan sebelumnya kerana telah berlaku perubahan pada kultivar padi, cuaca, alat taksonomi dan penghijrahan populasi. Oleh itu, tujuan kajian ini adalah untuk mengenal pasti dan mencirikan spesies *Fusarium* yang berkaitan dengan penyakit bakanae pada padi di Malaysia dan Bangladesh melalui pencirian morfologi, biologi, molekul dan fisiologi. Suatu tinjauan telah dibuat untuk penyakit bakanae di kawasan sawah padi di Malaysia dan Bangladesh pada tahun 2019-2022. Dua belas spesies *Fusarium* telah dikenal pasti secara morfologi dan biologi daripada 210 pencilan *Fusarium*. Walaubagaimanapun, berdasarkan pengecaman secara molekul menggunakan gen *TEF-1α* dan gen *RPB2*, 17 spesies *Fusarium* telah dikenal pasti iaitu *F. fujikuroi*, *F. verticillioides*, *F. proliferatum*, *F. andiyazi*, *F. mangiferae*, *F. longipes*, *F. solani*, *F. oxysporum*, *F. graminearum*, *F. commune*, *F. hainanense*, *F. bubalinum*, *F. pernambucanum*, *F. tanahbumbuense*, *F. sulawesiense*, *F. flagelliforme* dan *F. arcuatisporum*. Spektrum spesies *Fusarium* yang berkaitan dengan penyakit bakanae lebih luas di Malaysia berbanding dengan Bangladesh. Kemunculan *F. hainanense*, *F.*

bubalinum, *F. pernambucanum* dan *F. arcuatisporum* adalah rekod baharu di Malaysia, manakala *F. pernambucanum*, *F. tanahbumbuense*, *F. sulawesiense*, *F. flagelliforme* dan *F. commune* adalah baharu di Bangladesh. Ciri morfologi yang unik telah digunakan untuk pengecaman spesies *Fusarium* seperti pseudo-klamidospora, and telinga arnab yang mana ia dihasilkan oleh *F. andiyazi* dan *F. hainanense*. Kedua negara mempunyai populasi mengawan MP-A, MP-C, and MP-D. *Fusarium fujikuroi*, *F. verticillioides*, *F. proliferatum*, *F. andiyazi*, *F. longipes*, *F. graminearum*, *F. commune*, *F. bubalinum*, and *F. sulawesiense* adalah patogenik terhadap padi walaupun terdapat variasi simptom yang ketara. Postulat Koch telah dipenuhi dalam menentukan kepatogenan kulat tesebut. Berdasarkan kepada penanda mikrosatelit, variasi genetik bagi *F. fujikuroi* adalah tinggi dalam populasi berbanding di antara populasi Malaysia dan Bangladesh. Dua kluster genetik telah terbentuk dalam populasi Bangladesh disebabkan oleh fasa seksual. Kebanyakan isolate *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* menghasilkan asid gibberelik (GA_3) dan asid fusarik (FA). Ini merupakan laporan pertama yang membuktikan penghasilan FA oleh spesies *F. andiyazi*, *F. longipes*, *F. commune* dan *F. graminearum*, manakala GA_3 dihasilkan oleh *F. andiyazi*. Daripada penyelidikan ini, perhatian khusus perlu diambil terhadap spesies *F. proliferatum*, *F. andiyazi* dan *F. longipes* di Malaysia, dan populasi *F. fujikuroi* dan *F. commune* di Bangladesh. Berdasarkan hasil penyelidikan ini, strategi pengurusan untuk penyakit bakanae perlu mengambil kira ancaman daripada spesies *Fusarium* yang lain yang boleh memberi kesan kepada penghasilan padi yang mampan.

GENETIC DIVERSITY, PATHOGENICITY AND SECONDARY METABOLITES OF *Fusarium* spp. ASSOCIATED WITH BAKANAE DISEASE OF RICE IN MALAYSIA AND BANGLADESH

ABSTRACT

Bakanae disease is an emerging rice disease that has become a major problem in Asian rice growing countries, including Bangladesh and Malaysia. The disease can reduce rice production by up to 30%. *Fusarium fujikuroi* was known to cause bakanae disease, while only a few other *Fusarium* species were associated with it. The current population of *Fusarium* species may not be the same as previously reported due to changes in rice varieties, climate, taxonomic tool and population migrations. Therefore, the aim of the present study was to identify and characterise the *Fusarium* species associated with bakanae disease of rice in Malaysia and Bangladesh based on morphological, biological, molecular, and physiological traits. In 2019-2022, a survey for Bakanae-infected plants were conducted in different rice growing areas in Malaysia and Bangladesh. Accordingly, *Fusarium* isolates were isolated from Bakanae-infected plants. Twelve *Fusarium* species were morphologically and biologically identified from 210 *Fusarium* isolates. However, based on molecular identification using *TEF-1α* and *RPB2* genes, 17 *Fusarium* species, namely, *F. fujikuroi*, *F. verticillioides*, *F. proliferatum*, *F. andiyazi*, *F. mangiferae*, *F. longipes*, *F. solani*, *F. oxysporum*, *F. graminearum*, *F. commune*, *F. hainanense*, *F. bubalinum*, *F. pernambucanum*, *F. tanahbumbuense*, *F. sulawesiense*, *F. flagelliforme* and *F. arcuatisporum* were identified. Compared to Bangladesh, a broad spectrum of *Fusarium* species associated with Bakanae disease of rice was found in Malaysia. The presence of *F. hainanense*, *F. bubalinum*, *F. pernambucanum* and *F. arcuatisporum* are new records in Malaysia,

while *F. pernambucanum, F. tanahbumbuense, F. sulawesiense, F. flagelliforme* and *F. commune* are new in Bangladesh. Some unique morphological characters were used to identify the *Fusarium* species such as pseudo-chlamydospores, and rabbit ears were formed by *F. andiyazi, and F. hainanense,* respectively. Both countries contained the mating populations MP-A, MP-C and MP-D. *Fusarium fujikuroi, F. verticillioides, F. proliferatum, F. andiyazi, F. longipes, F. graminearum, F. commune, F. bubalinum,* and *F. sulawesiense* were pathogenic to rice, although there was considerable variation in symptoms. Koch's postulate was fulfilled to confirm the fungal pathogenicity. Based on microsatellite markers, the genetic variation of *F. fujikuroi* was higher within the population than between the populations in Malaysia and Bangladesh. Most isolates of *F. fujikuroi, F. proliferatum* and *F. verticillioides* produced gibberellic acid (GA_3) and fusaric acid (FA). It is the first report that FA is produced by *F. andiyazi, F. longipes, F. commune* and *F. graminearum* while GA_3 is produced by *F. andiyazi*. From these research, special concern should be taken to the species of *F. proliferatum, F. andiyazi* and *F. longipes* in Malaysia, and the population of *F. fujikuroi* and *F. commune* in Bangladesh. Based on the results of this study, the strategy to control bakanae disease should also consider the threat of other *Fusarium* species that could affect the sustainability of rice production.

CHAPTER 1

GENERAL INTRODUCTION

Rice (*Oryza sativa* L) is an important crop with great potential in terms of acreage and production in the world. Rice is the third most cultivated cereal after wheat and maize. It is grown on more than 163 million hectares of land worldwide with a yield of 769 million tonnes per year. Interestingly, more than ninety percent (90.5%) of the world's rice is grown by Asian farmers (FAOSTAT, 2020 http://www.fao.org/faostat/en/#data/QCL/visualise). Bangladesh is the third largest rice-growing country in the world and contributes significantly to global rice production. During rice cultivation, numerous biotic and abiotic stress factors occur due to the different ecological conditions. Biotic stress factors include diseases caused by various pathogens such as fungi, bacteria, viruses, and nematodes, which pose a serious threat to the sustainable production of rice. Rice diseases such as brown spot, blast, sheath blight, bakanae, and false smut has been caused by fungi.

The well-known seed borne disease is Bakanae, which has become an economic problem in the world's major rice-growing countries, including Asia, Africa, California and Italy (Ghazanfar et al., 2013). Bakanae disease of rice was first reported in Japan where it has been present since 1828 (Husna et al., 2020). Bakanae was considered a minor disease in the past that had no major impact on rice production. In recent years, the occurrence of bakanae disease has been reported in many rice-producing countries around the world. Now bakanae is an emerging disease in Asia, including Bangladesh (Gupta et al., 2015; Husna et al., 2020). The yield losses caused by bakanae range from 3.0 to 95% and depend on the region, the virulence of the fungus and the rice varieties used (Singh & Sunder, 2012; Gupta et al., 2015). The Bakanae disease incidence was 12.5% in Malaysia (Zainudin et al., 2008a). In

Bangladesh, bakanae disease has a significant impact on rice production, with yield losses of up to 25% (Husna et al., 2020).

Fusarium species can vary with geographical and climatic changes (Saremi & Saremi, 2013). Several *Fusarium* species, namely *F. fujikuroi, F. proliferatum, F. verticillioides, F. concentricum, F. anthophilum*, and *F. andiyazi* have been identified as the causal agents of bakanae disease (Amoah et al., 1995; Desjardins et al., 2000; Zainudin et al., 2008a; Amatulli et al., 2010; Prà et al., 2010; Choi et al., 2018). However, until 2013, *F. fujikuroi* was thought to be the predominant *Fusarium* species involved in the development of bakanae disease, although many other species have been isolated and identified from bakanae affected plants. More than one *Fusarium* species has been found to be capable of infecting rice and causing bakanae disease symptoms in Nepal (Desjardins et al., 2000). According to Hsuan et al. (2011), both *F. fujikuroi* and *F. proliferatum* can cause bakanae disease in rice plants. In Malaysia (Quazi et al., 2013) and Turkey (Eğerci et al., 2021), *F. proliferatum* was isolated and described as the causal pathogen of bakanae. In addition, *F. fujikuroi* and *F. moniliforme* (known as *F. verticillioides*) have been identified as the causative agent of Bakanae disease in India (Gupta et al., 2015; Puyam et al., 2019). Jeon et al. (2013) reported that *F. concentricum, F. verticillioides* and *F. proliferatum* are the potential causative agents of bakanae disease in Asian countries. Hossain et al. (2015a) identified *F. anthophilum* and *F. fujikuroi* as the causal agents of bakanae disease in South Africa. *Fusarium andiyazi* has also been associated with bakanae disease of rice in Italy and Turkey (Prà et al., 2010; Eğerci et al., 2022). Notably, the association of *Fusarium* species with Bakanae disease is still unclear, as different pathogens can cause variations in symptoms that may hinder the accurate identification of the disease. Two *Fusarium* species, namely *F. fujikuroi* and *F. proliferatum*, have frequently

caused bakanae disease in Malaysia (Zainudin et al., 2008a; Quazi et al., 2013). However, the association of *Fusarium* spp. with Bakanae disease of rice in Bangladesh is still unknown, although a few studies have been conducted on the phenotypic characteristics of *Fusarium* species causing Bakanae disease. It is believed that *F. fujikuroi* has been frequently associated with bakanae disease, but other *Fusarium* species can also cause this disease. Interestingly, the detailed information on this disease has hardly been extensively studied. The bakanae disease pathogen has several races, which makes it difficult to develop resistance varieties in breeding programmes. The *Fusarium* species associated with bakanae disease of rice need to be researched for effective control. Therefore, bakanae is a serious problem especially for rice farmers, industry and researchers.

The morphological identification of *Fusarium* species is mostly based on morphological characteristics, including macroscopic and microscopic features. The colour and appearance of the fungal colonies are the macroscopic features, while the size and shape of the microconidia, macroconidia and chlamydospores are the microscopic features (Nelson et al., 1992). These features were used to identify the *Fusarium* isolates to genus, or species level. However, molecular identification and phylogenetic analyses were considered for the isolates lacking conidia or similar features or other inconspicuous microscopic features (Hsieh et al., 2005). In particular, morphological features are very similar in some species, e.g. species within a species complex. Therefore, identification can often be incorrect if based solely on morphological characteristics.

The identification of *Fusarium* by morphology can be very confusing. Biological characterisation i.e. sexual compatibility, is one of the ways to reduce confusion, especially for species that have a sexual stage. Therefore, some *Fusarium*

species are differentiated based on sexual fertility by the biological species concept. The *Fusarium fujikuroi* Species Complex (FFSC), consists of 12 sexually fertile biological species (mating populations MP-A to MP-L) (Leslie, 1991; Klittich & Leslie, 1992; Kerényi et al., 1997; Steenkamp et al., 2000; Zeller et al., 2003). Three (3) mating populations of FFSC have been associated with Bakanae disease of rice, namely MP-C (anamorphic *F. fujikuroi*) was isolated from Taiwan rice (Hsieh et al., 1977), MP-A (anamorphic *F. verticillioides*) and MP-D (anamorphic *F. proliferatum*) were isolated from Asian rice. In addition, MP-A was isolated from rice from Africa, Australia and the USA (Voigt et al., 1995; Desjardins et al., 1997). Besides, MP-A (*F. verticillioides*), MP-B (*F. sacchari*), MP-C (*F. fujikuroi*) and MP-D (*F. proliferatum*) isolates were obtained from bakanae diseased rice plant in Malaysia. However, so far there is no report on the mating population of *Fusarium* species associated with Bakanae-infected rice in Bangladesh.

The identification of *Fusarium* species on the basis of morphological and biological characteristics is often difficult and time consuming (Leslie & Summerell, 2006). Therefore, phylogenetic analysis of *Fusarium* is performed to support morphological identification and evaluate the genetic relationship between closely related species (Jeon et al., 2013). The classification of fungal species at the DNA sequence level reveals the evolutionary and ecological relationships between different species (Mulè et al., 2005). Currently, gene sequencing of translation elongation factor 1-α (*TEF-1α*) and RNA polymerase II subunit (*RPB2*) of *Fusarium* species is commonly used to identify and characterise the fungus at the species level (Lima et al., 2021). The use of *TEF-1α* enables the correct identification of *Fusarium* species (O'Donnell et al., 1998a; Geiser et al., 2004; Kvas et al., 2009). The *TEF-1α* gene is highly informative and conservative at the species level and non-orthologous copies

of the gene did not occur. The *TEF-1α* gene is recommended for the identification of *Fusarium* species because this gene is present as a single copy with a high degree of sequence polymorphism between closely related species (Geiser et al., 2004). Therefore, *TEF-1α* is broadly applicable and receptive throughout the genus (Geiser et al., 2004). Recently, *RPB2* is the most useful marker for study of closely related *Fusarium* species. It appears to be a phylogenetically informative gene for the characterization of species in a species complex (Geiser et al., 2004). Phylogenetic analysis uses shared gene sequences to assess the evolutionary relationships of species, which are represented by a phylogenetic tree (Mirghasempour et al., 2022). Phylogenetic analyses of single and combined sequences are used to identify fungal isolates and confirm species identity, grouping isolates of the same species in the same clade and separating them from other species in different clades (Martin et al., 2010).

Fusarium is one of the most economically important genera of pathogenic species with a broad spectrum of hosts. They are filamentous, cosmopolitan and most commonly isolated fungi (Nelson et al., 1983; Burgess et al., 1994). *Fusarium* species, either saprophytic or endophytic, are found to be pathogenic when environmental conditions are suitable for disease development and are influenced by biotic and abiotic factors (Photita et al., 2004; Schulz & Boyle, 2005; Bacon et al., 2008). Therefore, a pathogenicity test is required to confirm whether the *Fusarium* species associated with Bakanae disease are pathogenic or non-pathogenic.

The composition and genetic diversity of fungal populations is influenced by the distribution of mating types and sexual fecundity. Mating type primers, simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP) and microsatellites have been to assess the genetic diversity of fungal populations. By using mating type primers, some genotypic variation was detected in the *F. fujikuroi*

population in California (Carter et al., 2008). The genetic variability of *F. fujikuroi* populations was investigated in Italy using 19 polymorphic SSRs (Valente et al., 2017). A set of newly developed polymorphic SSR markers was used to analyse *F. fujikuroi* isolates collected from different cities in Taiwan to determine the genetic diversity of *F. fujikuroi* (Chen et al., 2016). In India, isolates of *F. fujikuroi* from diseased plants were analysed for molecular variability by using universal primers (Bashyal et al., 2020). It is crucial to study the genetic diversity of a pathogen in order to control it effectively.

Fusarium species have a specific profile of secondary metabolites (Thrane, 2001). A variety of secondary metabolites such as fusaric acid, gibberellins, moniliformin, fumonisins, fusaroproliferin, and beauvericin are produced by species within the FFSC (Desjardins & Proctor, 2007; Zainudin & Perumal, 2015). In addition, the production of secondary metabolites varies between closely related species of FFSC. Therefore, the production of a particular metabolite is informative for phylogenetic characterisation and species identification. The ability to produce certain secondary metabolites has implications for the evolution and ecological adaptations of fungi. Gibberellin is considered a virulence factor of *F. fujikuroi*. Fusaric acid positively associated with the increase in disease symptoms of bakanae (Quazi et al., 2016). Interestingly, there were no previous studies on physiological studies on secondary metabolite profiles (GA_3 and FA) in evaluating the pathogenesis of *Fusarium* isolates isolated from bakanae infected rice in Bangladesh.

Rice is the most important staple food in Malaysia and Bangladesh. Several *Fusarium* species, namely *F. fujikuroi*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides* have been isolated from infected rice plants in Malaysia (Zainudin et al., 2008a). In Bangladesh, the relationship between *Fusarium* species

and bakanae disease of rice is still unclear. Every year, a significant number of Bangladeshis migrate to Malaysia to work as labourers in many industries including agriculture. They may carry pathogens on their shoes, bags, and other belongings. Therefore, the population composition of *Fusarium* species could change or the genetic variation of *Fusarium* isolate could have been caused. The movement of people between these two countries could result in the mixing of *Fusarium* spp. in Malaysia. However, information on the genetic variation of *Fusarium* species associated with bakanae disease of rice in Malaysia and Bangladesh, and the pathogenicity of these species are very limited. Few data are available on *F. fujikuroi* populations associated with bakanae disease, including data from India (Bashyal et al., 2020), California (Carter et al., 2008), Italy (Valente et al., 2017), and the Philippines (Cumagun et al., 2011; Cruz et al., 2013).

Therefore, the present study was carried out to achieve the following objectives:

i. To determine the *Fusarium* species composition from rice bakanae disease in Malaysia and Bangladesh.

ii. To assess the pathogenicity of selected isolates towards rice.

iii. To evaluate the genetic variability of the *Fusarium* isolates isolated from bakanae disease in Malaysia and Bangladesh.

iv. To analyse and quantify the secondary metabolite profiles gibberellic acid (GA_3) and fusaric acid (FA) produced by selected *Fusarium* isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 Rice (*Oryza sativa*)

Rice (*Oryza sativa* L., Poaceae) is an important cereal originally from India and China (Crawford & Shen, 1998; Gnanamanickam, 2009). Rice is cultivated in different geographical regions ranging from the tropics to temperate zones (Chin & Supaad, 1986). Moreover, *O. indica* and *O. japonica* are the two major races of *O. sativa* in tropical areas. Rice is one of the most important food crops in the world, after wheat and maize. With a cultivated area of around 163 million hectares and a production of approx. 769 million tonnes/year (FAOSTAT, 2020, http://www.fao.org/faostat/en/#data/QC/visualise), it is widespread. More than 90% of the world's rice production is grown and consumed in Asian countries. Rice plays important role in the human diet as it contains protein, carbohydrates, minerals, and vitamins. It provides 21% energy and 15% protein, minerals, vitamins, and fibre (Maclean et al., 2002). The demand for rice has increased in line with population growth.

2.1.1 Status of rice in Malaysia

Rice is the most important staple food in many Asian countries, including Malaysia (Ariffin & Nik Fuad, 2003) and Bangladesh (Shelley et al., 2016). Rice has a long and distinguished history in Malaysia plays an essential role in daily life. Rice was cultivated as early as the 14th century in Terengganu (Hill, 1977) and in the early 16th century in Kedah, Perak, Melaka, Johor, and Pahang (Chin &Supaad, 1986). Malaysia ranks 27th in rice production in the world. In Malaysia, rice production reached 2.35 million tonnes on 644,908 hectares of cultivated land, with an average yield of 3.6 tonnes per hectare in 2020 (FAOSTAT, http://www.fao.org

/faostat/en/#data/QCL). Rice production in Malaysia covers about 65% of the population's needs (Hussin et al., 2020). Malaysia needs to grow more rice to meet the needs of its rapidly growing population demands and achieve self-sufficiency. Diseases are a constant obstacle to increasing rice production in Malaysia. Malaysia imports rice from Pakistan, India, Vietnam and Thailand.

2.1.2 Status of rice in Bangladesh

Millions of people around the world rely on rice as one of the most common staple foods to meet their daily needs. Bangladesh is currently the third largest rice-producing country in the world after China and India. In Bangladesh, rice is grown on 11.42 million hectares, which accounts for about 75% of the cultivated area and a total of 54.9 million tonnes are produced (FAOSTAT, 2020 https://www.fao.org /faostat/en/#data/QCL). The rice sector contributes 50% to agricultural GDP and 16% to national income. However, cultivation often encounters various obstacles, such as unfavourable weather conditions, the planting of certain specific rice varieties outside the country and the occurrence of diseases and pests.

2.2 *Fusarium* genus

Fusarium is a well-researched genus that has been extensively studied at all levels, including genetic mechanisms, toxin production, pathogenicity, biodiversity, and evolution. *Fusarium* is a fungus belonging to the phylum Ascomycota in the class Sordariomycetes and the order Hypocreales. It was first described by Link (1809) as *Fusisporium*. The most important classification study was carried out by Wollenweber & Reinking (1935). In fact, some *Fusarium* species go through a in sexual (teleomorphic) stage and therefore the nomenclature is changed to *Gibberella, Nectria,* or *Haemonectria* (Leslie & Summerell, 2006). In 2011, the International Code of

Nomenclature for algae, fungi and plants (ICNafp) changed the name, although this double nomenclature of anamorph and teleomorph is not used at this time (Aoki et al., 2014). Therefore, anamorph of *Fusarium* (asexual names) is formally used for species with sexual stages, replacing the names *Gibberella*, *Nectria* and *Haemonectria*. This genus has numerous members that occur as pathogens, endophytes and saprophytes in plants and soils throughout the world (Geiser et al., 2004). Most of these members have been identified as phytopathogens. *Fusarium* species can be found in soil, water, air, and other substrates. It is one of the most diverse and widespread phytopathogenic fungi, causing economically important rot, wilt and canker in a wide range of field, horticultural, ornamental and forest crops, agricultural products and natural ecosystems. *Fusarium oxysporum* has a broad host range and infects both monocotyledonous and dicotyledonous plants, while *F. graminearum* and *F. verticillioides* have a narrow host range and infect cereal crops. *Fusarium* also produces a variety of toxic secondary metabolites known as mycotoxins, (fusaric acid, fumonisins and trichothecenes), which contaminate agricultural products and make them unfit for consumption (Matny, 2015).

Domain: Eukaryota

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Nectriaceae

Genus: *Fusarium*

2.3 Rice Bakanae Disease (RBD)

2.3.1 Historical background, distribution and economic loss

Bakanae, one of the oldest known rice diseases, was discovered by Japanese scientists almost 100 years ago. Bakanae disease of rice was first described by Hori (1898), but it was found in Japan as early as 1828 (Gupta et al., 2015). Kurosawa (1926) described the effect of the pathogen on the plant and discovered that the pathogen produces a chemical that stimulates stem elongation and suppresses chlorophyll content in infected plants. Following this discovery, scientists from various fields, including plant physiology and biochemistry, endeavoured to identify the chemical responsible for disease development. As a result, the chemical called gibberellin from *F. moniliforme*, which causes bakanae disease, was identified (Yabuta & Hayashi 1939).

The Japanese word 'bakanae' means bad or foolish seedlings. Bakanae has many names in many countries of the world such as Fusarium blight, elongation disease, fusariosis, white stalk, palay lalake, otoke nae, equatorial foot rot, foolish plant or foot rot (Singh & Sunder, 2012). It is also known as white head and root rot disease (Saremi et al., 2008).

Bakanae disease is widespread in rice-growing areas all over the world (Figure 2.1). Bakanae disease has been reported from most rice-producing countries, including Japan (Ito & Kimura, 1931), India (Thomas, 1931), China (Yang et al., 2003), Taiwan (Chen et al., 2016), Philippines (Reyes, 1939), Thailand (Kanjanasoon, 1965), South Korea (Park et al., 2009), Bangladesh (Miah & Zaman, 1973), southern Spain (Marin-Sanchez & Jimenez-Diaz, 1982), Nepal (Desjardins et al., 2000), Italy (Amatulli et al., 2010), Malaysia (Zainudin et al., 2008a), Indonesia (Zainudin et al., 2008a), North America (Prà et al., 2010), Australia (Heaton & Morschel, 1965) and Pakistan (Bhalli,

2001). The disease has spread to entire countries such as India, Bangladesh, China, Nepal, and Laos in Asia, Guyana and Suriname in South America, and Australia.

Figure 2.1 Bakanae disease occurring countries throughout the world (www.cabi.org/Dmpp)

Bakanae was one of the first scientifically described rice diseases. Yield losses due to bakanae range from 3.0-95.4%, depending on the area under cultivation, fungal isolates, virulence and varieties grown (Table 2.1). This disease also significantly deteriorates the quality of rice (Bashyal & Aggarwal, 2013). Bakanae disease is now an emerging concern in both India and Bangladesh. In Bangladesh, yield losses of up to 25% have been reported in susceptible rice cultivars (Husna et al., 2020). In India, the disease is particularly prevalent in basmati rice cultivars, with yield losses ranging from 15-25% reported in states such as Uttar Pradesh, Assam, Andhra Pradesh, Tamil Nadu, Haryana, and Punjab (Gupta et al., 2015).

Table 2.1 Yield losses caused by bakanae disease in various countries

Country	Distribution	Yield loss	Reference
Japan	Present	20 – 50%	Ito & Kimura (1931)
Thailand	Present	3.7-14.7%	Kanjanasoon (1965)
Nepal	Present	40%	Desjardins et al. (2000)
Philippines	Present	1-13%	Reyes (1939)
Pakistan	Present	10-50%	Bhalli (2001)
Bangladesh	Widespread	25%	Hossain et al. (2011); Hossain et al. (2013, 2015b)
Turkey	Present	10-15%	Surek & Gumustekin (1994)
India	Widespread	15-25%	Pavgi & Singh (1964); Singh & Sunder (2012)
Australia	Widespread	70%	Heaton & Morschel (1965)
America	Present	-	Carter et al. (2008)
Iran	Present	75%	Saremi et al. (2008)
Malaysia	Present	-	Zainudin et al. (2008a)
Macedonia	Present	2% - 20%	Karov et al. (2009)

2.3.2 Etiology

Accurate identification of the aetiological agent is crucial for infection control and disease management (Wingfield et al., 2012). Hori (1898) determined that *F. heterosporum* Nees was the causative fungus. *Gibberella fujikuroi* is the name of the perfect stage. Booth (1971) identified the anamorph as *F. moniliforme*, while Nirenberg (1976) distinguished it as *F. fujikuroi*, a separate species. Nelson et al. (1983) did not recognise *F. fujikuroi* as a separate species, but assigned it to *F. moniliforme* as a "short chain" species of *F. moniliforme*. According to Marasas et al. (1986), polyphialides were found in isolates of *Fusarium* from rice plants diseased with Bakanae ("Bakanae isolates") and excluded from *F. moniliforme*. They noted that they used the name *F. fujikuroi* (Nirenberg, 1976) for these cultures. Tiedt & Jooste (1988) found that *F. fujikuroi* can be clearly distinguished from *F. moniliforme* (*F. verticillioides*) based on studies of the ultrastructure of colony development. They came to the conclusion that *F. fujikuroi* can be distinguished from *F. moniliforme* (*F. verticillioides*), but not from *F. proliferatum. Fusarium proliferatum* is phylogenetically closely related to the rice pathogen of *F. fujikuroi* (teleomorph, *G.*

fujikuroi; mating population C) (O'Donnell & Cigelnik, 1997; O'Donnell et al., 1998a).

Based on morphological characteristics, earlier phytopathologists believed that *F. moniliforme* (renamed *F. fujikuroi*) was the only species involved in the bakanae disease complex (Snyder & Hansen, 1945; Nirenberg, 1976; Nelson et al., 1983). Webster & Gunnell (1992) and Desjardins et al. (2000) suggested that one or more *Fusarium* species cause rice Bakanae disease and the developing symptoms of bakanae disease. Accordingly, several *Fusarium* species were isolated and identified from bakanae affected plants. However, until 2013, *F. fujikuroi* was the predominant pathogen responsible for bakanae development. Carter et al. (2008) identified *F. fujikuroi* as the causal agent of bakanae disease of rice in the USA. Amatulli et al. (2010) identified several *Fusarium* species associated with rice diseases and showing bakanae symptoms in Italy. However, pathogenicity studies determined that *only F. fujikuroi* can infect rice plants and cause bakanae. According to Hsuan et al. (2011), both *F. fujikuroi* and *F. proliferatum* are associated with Bakanae disease in rice. *Fusarium proliferatum* has been isolated and reported as the causal agent of bakanae in Malaysia (Quazi et al., 2013) and Turkey (Eğerci et al., 2021). Phylogenetically, *F. proliferatum* and *F. fujikuroi* belong to the Asian clade of GFSC (O'Donnell et al., 1998a) and are very closely related species although they have different karyotypes (Leslie & Summerell, 2006). *Fusarium fujikuroi, F. proliferatum*, and *F. verticillioides* (*F. moniliforme*), *F. concentricum* have been associated with bakanae disease of rice in India as well as Asian countries (Wulff et al., 2010; Hsuan et al., 2011; Jeon et al., 2013; Bashyal, 2018). *Fusarium andiyazi*, was identified in African sorghum (Marasas et al., 2001), and associated with bakanae disease of rice (Prà et al., 2010; Wulff et al., 2010; Eğerci et al., 2022). In Africa (Hossain et al., 2015a), *F. anthophilum* and *F.*

fujikuroi caused bakanae disease in rice. The infection rates of bakanae disease are between 0.25% and 20%, caused by *F. fujikuroi*, *F. proliferatum*, *F. andiyazi*, and *F. verticillioides*. Of these, *F. fujikuroi* is restricted to Asia, while the other three are found in Asia and Africa.

2.3.3 Disease symptoms

Bakanae disease of rice has produced complex disease phenotypes. The disease shows a range of symptoms, such as crown and root rot, abnormal stem elongation, wilting, stunting, and adventitious root growth at nodes in the lower parts of the stems (Webster & Gunnell, 1992). The presence of numerous pathogens leads to variations in symptoms, making identification of the disease difficult. However, it is not clear that the *Fusarium* species are associated with the different symptoms of the disease. According to Wulff et al. (2010), African and Asian populations of *Fusarium* species (*F. fujikuroi* species complex) can reduce seed germination and cause various symptoms in rice. *Fusarium andiyazi*, which is associated with bakanae disease of rice, produces general symptoms of seedling wilt, i.e. a reduction in root length and discoloration of the roots. In particular, symptoms may vary depending on the fungus and rice variety.

The symptoms of bakanae are mainly categorized into two types, namely hyper-elongation or hypo-elongation of the stem (Niehaus et al., 2017). The first symptom of bakanae: the infected seedlings are more elongated, thinner, and slightly chlorotic compared to healthy seedlings (Figure 2.2A). The fungus causes the rapid growth of infected plants by producing gibberellin, a plant hormone. Bakanae plants are often seen above healthy rice plants. The second symptom is stunting of the rice plants (Figure 2.2B). The stunted of rice plants is due to crown rot (Amoah et al., 1995). In addition to the typical bakanae symptoms, several isolates or isolates cause

stunting of rice (Jeon et al., 2013; Hur et al., 2015). However, the development of symptoms depends on the amount of inoculum, the pathogen isolates and the production of gibberellic acid and fusaric acid, which lead to stunting. In severe infection, white mycelia and pink-coloured sporodochia can be seen on the stem just above the water level.

Although bakanae is a seedling disease, it can occur during the entire growth stage. The disease can infect rice plants from the early to the mature stage, even infected rice seeds lead to poor germination or wilting (Iqbal et al., 2011). Sometimes the bakanae symptoms become inconspicuous after transplanting the infected seedlings to the field and the symptoms may appear at tillering stage. Infected seedlings tend to die gradually from the seedling stage to maturity. The sterile panicles are noticed when the infected plants survive to the heading stage. The flag leaf is characterized by its raised, more horizontal position on mature plants. In older plants, the roots, crowns, stems, leaf sheaths and panicles may be infected. Heavily infected grains may be discoloured (Amatulli et al., 2010).

Figure 2.2 Bakanae disease symptoms in the plant house, USM. (A) Elongated rice seedling; (B) Stunted rice seedling

2.3.4 Disease cycle and disease development

Bakanae is a monocyclic seed borne disease. The main source of infection is the seed. Infested seed is the main source of inoculum, which spreads the disease in both infested and non-infested fields. The conidia are mainly spread by wind and water (Figure 2.3). The spread of the disease has also been observed when diseased and healthy seedlings were planted alternately in a row (Singh et al., 2019). The seeds are infected by airborne ascospores produced during flowering or by contamination of the seeds with conidia at harvest (Sun, 1975). The conidia are produced in sporodochia on infested stems and can be transferred to flowers of neighbouring plants by water splashes and wind. The pathogen survived in the endosperm and embryo at 15.00 and 6.25%, respectively (Kumar et al., 2016a). The fungus can transmit the disease to the subsequent plant generation and to different locations via the seed trade, where airborne conidia can contaminate the seed at harvest. The fungus spreads vigorously on the stems of infected plants near the water level resulting in a visible fungal growth of pink to white colour at the base of the stem after the water is drained. This cotton-

like fungal growth produces millions of conidia at harvest and contaminates the healthy seeds. Infection can also occur from mycelia and spores that remain in the water used to soak the seeds.

The fungus can overwinter in infected seeds, crop residues and soil (Cartwright et al., 2018). The fungus is carried both inside and, on the outside of seeds, and the fungus recovered more in lemma than in palea (Kumar et al., 2015). Disease development reached 8.5% in artificially inoculated soil (Singh et al., 2019). The soil inoculum is not very significant as it has a relatively short life span (Watanabe, 1974; Singh & Sunder, 2012), i.e. the fungus can survive for a limited time in the soil via conidia, ascospores, thick-walled hyphae or chlamydospores. According to Sun (1975), the pathogen can survive in the soil for 3-4 months in the form of macroconidia or thick wall hyphae. Puyam (2017a) also investigated that the Bakanae pathogen is weak as a soil inhabitant and the survival of the pathogen decreases with time in the field environment. Furthermore, the pathogen can survive in infected debris for 10 to 28 months and serve as a source of inoculum (Sunder & Satyavir, 1997). Bakanae disease is mainly caused by the pathogen in seeds or infected plant debris in the soil (Biswas & Das, 2003).

The occurrence and establishment of *Fusarium* species as plant pathogens depends on geography and climate (Castellá & Cabañes, 2014). The development of bakanae disease symptoms is influenced by various abiotic factors such as temperature, humidity etc. In addition, various soil properties such as soil moisture, temperature, pH and nitrogen fertilizer are involved in the development of Bakanae disease (Nyvall, 1999). The elongation symptoms were more likely to occur at high moisture and the rot symptoms were more likely to occur at low soil moisture (Yadav et al., 2020). Environmental conditions play a key role in the development of

symptoms. At temperatures up to 35 °C and high humidity, stem elongation occurs; at low relative humidity, plants can become stunted (Naeem et al., 2016; Matic et al., 2017). The incidence of bakanae was high during the summer season and high temperatures and relative humidity affected the development of the disease. Bakanae was found to be more prevalent in upland rice fields than in flooded areas (Hashioka, 1971). In addition, the application of high doses of nitrogen fertilisers reduced the pathogen population in the soil (Mandal & Chaudhuri, 1988). More disease was observed in the paddy fields grown by transplanting method than by broadcasting method (Saremi & Farrokhi, 2004). The development of bakanae disease symptoms also depends on the balance of toxin and growth regulators (Amoah et al., 1995). The fungus is able to produce both gibberellin and fusaric acid, so that the seedlings grow in the early stage and are stunted in the later stage. Ascospores and conidia remain attached to the seeds during germination and infect the seedlings via the crown and roots. Conidial germination of the pathogen was highest in the root tissue, followed by the stem. Internal infection of the seeds occurs only in a few cases. Heavily infected seeds are discoloured, resulting in stunted seedlings. Typical bakanae symptoms were observed in seedlings from infected seeds without discolouration.

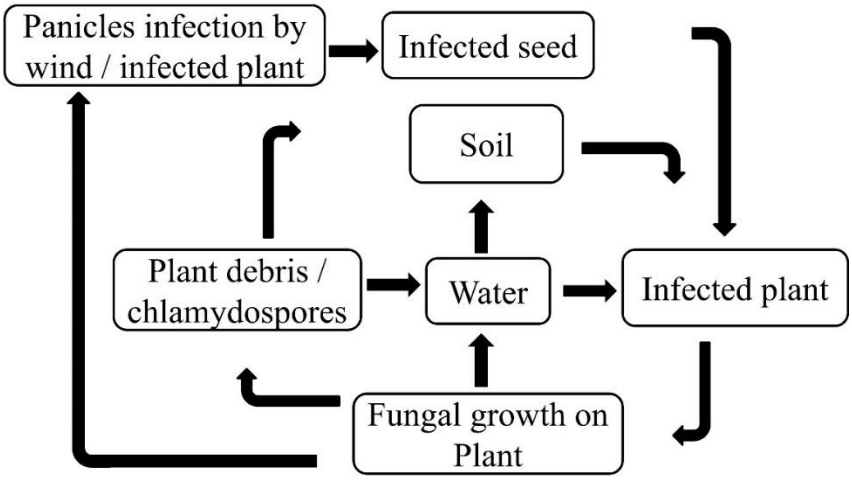

Figure 2.3 Disease cycle of bakanae disease

2.4 *Fusarium* spp. associated with bakanae disease

Fusarium fujikuroi is usually well-known as the causal pathogen of bakanae disease. In fact, several *Fusarium* (*F*.) species were isolated and identified from bakanae infected rice plants and seeds, for instance, six species of *Fusarium* viz. *F. anthophilum, F. chlamydosporum, F. compactum, F. equiseti, F. fujikuroi* and *F. semitectum*. Among them, *F. anthophilum* and *F. fujikuroi* were found to cause bakanae disease of rice in South Africa (Hossain et al., 2015a). In addition, *F. fujikuroi, F. proliferatum, F. sacchari, F. subglutinans* and *F. verticillioides* were isolated from rice plants exhibits typical bakanae symptoms in Malaysia and Indonesia (Zainudin et al., 2008a). Again, *F. fujikuroi, F. concentricum, F. proliferatum*, and *F. verticillioides*, were found in rice seeds in many Asian countries (Jeon et al., 2013). Other *Fusarium* species reported from rice plant parts includes *F. andiyazi, F. graminearum, F. equiseti, F. oxysporum* and *F. persicinum* (Amoah et al., 1995; Desjardins et al., 2000; Hsuan et al., 2011; Kim et al., 2012a; Mohiddin et al., 2021). Understanding the association of *Fusarium* population with a disease is essential for developing and implementing effective management strategies. Accurate identification of the etiological agent is essential to disease management and infection control (Wingfield et al., 2012). Therefore, the *Fusarium* spp. associated with Bakanae disease of rice must be accurately identified. Three methods are generally used to identify *Fusarium* species associated with bakanae disease of rice, namely morphological, biological, and molecular identification.

2.4.1 Morphological characterization

Morphological identification is a widely used approach to identification in which the characteristics of the fungus (phenotypic features) are described and the fungal isolates are classified into genera and species. In morphological identification,

the similarities and dissimilarities of the fungal characteristics are determined in suitable culture media. Morphological characterisation of *Fusarium* species is based on size, shape, formation of macroconidia, microconidia, conidiophores, presence or absence of chlamydospores, culture pigmentation, growth, morphology and diameter of the colony on PDA and its colour (Zainudin et al., 2008a; Hwang et al., 2013; Yadav et al., 2014). The morphological identification of *Fusarium* consists of macroscopic and microscopic features. Microscopic features such as the shape and size of the conidia, types of conidiogenous cells, and the chlamydospores are among the important features for the identification of *Fusarium* species (Leslie & Summerell, 2006).

The most commonly used macroscopic features to identify *Fusarium* are pigmentation (colour of the back side plate of the colony) and the appearance of mycelia growing on an agar plate. These are usually used to confirm a diagnosis based on other characteristics rather than for initial identification. Growth rates can be determined by linear growth in a test tube or by radial growth in a petri dish. In addition, secondary characteristics can be physiological data on toxins and other metabolites produced. However, these data are not available for routine diagnosis (Summerell et al., 2003).

Microscopic features are the most important features for the morphological identification of *Fusarium* species. Microscopic features include the shape and size of the conidia, the types of conidiogenous cells and the chlamydospores (Leslie & Summerell, 2006). The characteristics of the macroconidia are important for the identification of *Fusarium* species. Macroconidia are found in sporodochia on Carnation Leaf Agar (CLA) and they are uniform in shape and size. In addition, the number of septate and the shape of the apical and basal cells in macroconidia are

important characteristics. In contrast, microconidia are usually abundant and diverse and are more frequently found in growing hyphae (Leslie & Summerell, 2006). In microconidia, the difference between the two conidogenous cells of monophialids and polyphialids, the microconidial chains, can be clearly recognised under the microscope. Chlamydospores are located in the hyphae either on top of or below the agar. Chlamydospores are structured singly, in pairs, in chains or clumps and have either rough or smooth walls (Summerell et al., 2003), which is can easily be confused with pseudochlamydospores and swollen cells.

Defining a species on the basis of morphological characteristics is challenging for some *Fusarium* species that have overlapping characteristics, different morphology, mutation, isolates of the same species, cultural variation etc. (Leslie & Summerell, 2006; Kvas et al., 2009). Nevertheless, this method is often difficult and usually requires a great understanding of classical taxonomy (Capote et al., 2012). Therefore, morphological identification is not always recommended to identify *Fusarium* species because of the possibility of misidentification due to the presence of cryptic species especially among members of the FFSC, FIESC (Lucking et al., 2014) which are morphologically similar and have few or ambiguous features to distinguish them. In addition, cultural differences between isolates of the same species have been reported (Leslie et al., 2001; Summerell & Leslie, 2011).

2.4.2 Biological characterization

Biological species or mating populations (MPs) is additional feature used to overcome the taxonomic difficulties. Biological species are characterised by the fact that two different individuals of the same species can interbreed, resulting in the production of viable offspring (Mayr, 1963). In biological identification, isolates capable of interbreeding are categorised within the same mating population. This

approach is particularly applicable to species that exhibit a perfect or teleomorphic stage (Leslie, 1995).

Most *Fusarium* species are heterothallic, i.e. they are self-sterile. Their sexual reproduction process is controlled by two alleles located at a single mating type locus. Individuals within the same mating population can reproduce sexually among themselves, but are unable to be sexual fertile with individuals from other mating populations (Leslie et al., 2001). The *Gibberella fujikuroi* Species Complex (GFSC) comprises 12 sexually fertile biological species, which are referred to as mating populations MP-A to MP-L (Leslie, 1991; Klittich& Leslie, 1992; Kerényi et al., 1999; Steenkamp et al., 2000; Zeller et al., 2003).

Three mating populations have been identified within the GFSC that are associated with Bakanae disease in rice. Mating population C (MP-C) (anamorph *F. fujikuroi*) was detected in isolates from Taiwan (Hsieh et al., 1977). Mating population-A (anamorphi *F. verticillioides*) and mating population-D (anamorph *F. proliferatum*) were obtained from Asian rice, while MP-D was found in African, Australian and American rice (Voigt et al., 1995; Desjardins et al., 1997). In addition, MP-A, MP-B, MP-C, and MP-D were found on samples of Bakanae infected rice plants in Malaysia and Indonesia (Zainudin et al., 2008b).

Crossing is an effective method of exchanging genetic material, combining genotypes and obtaining mutation on a large scale, although crossbreeding is not always successful (Latiffah et al., 2011). The unequal distribution frequency of both mating types within the population of *F. fujikuroi* suggests that asexual reproduction predominantly contributes to the propagation of isolates under field conditions. However, the coexistence of both mating types in *F. fujikuroi* indicates the potential of the population for sexual reproduction (Bashyal et al., 2020).

Certain *Fusarium* species, such as *F. graminearum*, are homothallic, i.e. they are self-fertile. This characteristic enables these species to reproduce sexually without the need for a partner (Leslie & Summerell, 2006). Therefore, biological identification is not possible for homothallic species and those that only have an asexual stage.

2.4.3 Molecular characterization

Molecular techniques are used for the detection and recording of fungi. This technique is also used to identify unknown species of fungi. This technique is used as a tool to obtain information on genetic relationships, taxonomy and epidemiology related to fungi (Refai & Hassan, 2015). Two methods are generally used in the identification of fungi, namely DNA barcoding based on a specific gene or region and sequence alignment of one or more genes using the phylogenetic approach. To identify unknown isolates, the DNA sequence of a gene or region is compared with sequence databases such as the National Centre for Biotechnology Information (NCBI) or FUSARIUM-ID, and species identity is based on sequence similarity determined using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Another method for species identification is the phylogenetic approach, in which the unknown isolates are placed in a group generated from the evolutionary tree (Brinkman &Leipe, 2001).

DNA sequence data are currently being used extensively to explain the phylogenetic species within the species complex (O'Donnell et al., 1998a.b). DNA sequencing of a particular gene or region is often used to identify fungi to verify genetic variation. DNA sequence data are regularly used for rapid and reliable identification of species. In phylogenetic analysis, DNA sequences are used to distinguish *Fusarium* species with similar morphological characteristics. In addition, the isolates in a species complex such as FFSC, FIESC and FSSC are distinguished