IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM PADDY, SUGARCANE AND MAIZE

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IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM PADDY, SUGARCANE AND MAIZE

by

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LIST OF SYMBOLS

µL	Microliter
µM	Micromole
µm	Micrometer
bp	Base pair
cm^2	Square centimeter
g	Gram
h	Hour
Ha	Hectares
$Kgcm^{-2}$	Kilogram per square centimeter
L	Liter
mA	Milliampere
min	Minute
mL	Microliter
mm^2	Millimeter square
Mt	Metric tan
rpm	Revolutions per minute
s	Second
V	Volt

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
aLRT	approximate Likelihood Ratio Test
BLAST	Basic Local Alignment Search Tool
CLA	Carnation Leaf Agar
CA	Carrot Agar
CNI	Close-Neighbor-Interchange
CM	Complete Medium
DAF	DNA Amplification Fingerprinting
DNA	Deoxyribonucleic acid

dNTP	deoxynucleoside triphosphate
FAO	Food and Agriculture Organization
FB	Fumonisin B
HMG	High Mobility Group
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer
MAT	Mating type
MEGA	Molecular Evolutionary Genetic Analysis
ML	Maximum Likelihood
MP	Maximum Parsimony
mtSSU	Mitochondrial small subunit
MON	Moniliformin
NJ	Neighbor Joining
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PHYML	Phylogentic Maximum Likelihood
PPA	Peptone Pentachloronitrobenzene Agar
PSA	Potato Sucrose Agar
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SMC	Simple Matching Coefficient
Spp.	Species (plural)
Syn.	Synonym
TBE	Tris-borate-EDTA
TEF-1α	Translation Elongation Factor-1 alpha
UPGMA	Unweighted Pair-Group Method with Arithmetic mean
Var.	Variation
VCG	Vegetative Compatible Group
WA	Water agar
ZEA	Zearalenone

PENGENALPASTIAN DAN PENCIRIAN SPESIES *Fusarium* DARIPADA PADI, TEBU DAN JAGUNG

ABSTRAK

Kebanyakan penyakit pada padi, tebu and jagung disebabkan oleh spesies *Fusarium* patogenik daripada ahli seksyen Liseola atau spesies kompleks *Gibberella fujikuroi*, yang sukar dikenalpasti dan dicirikan dengan hanya menggunakan satu kaedah kajian. Oleh itu, kajian morfologi, pengawanan dan analisis molekul telah dijalankan untuk mengenalpasti, membuat pencirian dan memerhatikan variasi genetik pencilan *Fusarium* yang dipencilkan dari tiga perumah iaitu padi, tebu dan jagung. Sejumlah 86 pencilan *Fusarium* digunakan dalam kajian ini yang terdiri daripada 36 pencilan yang telah berjaya dipencilkan daripada padi, tebu dan jagung yang berpenyakit manakala 50 pencilan adalah daripada padi dan tebu diperolehi daripada kultur stok. Enam spesies *Fusarium* iaitu *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* dan *F. verticillioides* telah dapat dikenalpasti melalui ciri-ciri. Gabungan tindakbalas berantai polimerase teknik pembatasan kepanjangan pecahan polimorfisme (PCR-RFLP) kawasan penjarak intergen (IGS) menggunakan 10 enzim pembatasan iaitu *Alu* I, *Bsu* 15I, *Bsu* RI, *Eco* 88I, *Eco* RI, *Hin* 6I, *Hin* fI, *Msp* I, *Rsa* I dan *Xho* I digunakan untuk pencirian pencilan-pencilan *Fusarium*. Jalur pembatasan yang dihasilkan adalah sangat bervariasi dan analisa kluster UPGMA menunjukkan secara amnya pencilan daripada spesies yang sama dikelompokkan dalam kluster yang sama. Berdasarkan kajian pengawanan, hanya 60.5% daripada pencilan *Fusarium* berjaya mengawan dengan sekurang-kurangnya salah satu daripada lima

populasi kacukan iaitu A (*G. moniliformis*), B (*G. sacchari*), C (*G. fujikuroi*), D (*G. intermedia*) dan E (*G. subglutinans*). Daripada kajian filogenetik menggunakan gen penterjemahan pemanjangan faktor-1 alfa (TEF-1α), enam spesies *Fusarium* iaitu *F. andiyazi*, *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. sacchari* dan *F. verticillioides* telah dikenalpasti. Ketiga-tiga topologi pohon filogenetik yang dihasilkan oleh "neighbor-joining" (NJ), parsimoni maksimum (MP) dan "maximum likelihood" (ML) adalah hampir sama di mana pencilan yang mempunyai identiti yang sama, berkelompok dalam kumpulan yang sama. Secara keseluruhannya, identiti pencilan-pencilan spesies *Fusarium* dari seksyen Liseola dapat ditentukan menerusi jujukan gen TEF-1α, dan keputusan dari kajian morfologi dan pengawanan boleh digunakan sebagai maklumat tambahan dalam mengenalpasti dan pencirian spesies *Fusarium* seksyen Liseola.

IDENTIFICATION AND CHARACTERIZATION OF *Fusarium* SPECIES FROM PADDY, SUGARCANE AND MAIZE

ABSTRACT

Most of the plant pathogenic *Fusarium* spp. associated with paddy, sugarcane and maize are members of section Liseola or *Gibberella fujikuroi* species complex which mostly could not be identified or characterized accurately by only using one approach. Hence, morphological, mating studies and molecular analysis were applied in this study to identify, characterize and to observe the genetic variability of *Fusarium* spp. from three hosts, paddy, sugarcane and maize. A total of 86 isolates of *Fusarium* were used in the present study which comprised 36 isolates successfully isolated from diseased paddy, sugarcane and maize and 50 isolates from paddy and sugarcane from the stock culture. Six species of *Fusarium* namely, *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. sacchari*, *F. subglutinans* and *F. verticillioides* were identified by using morphological characteristics. Polymerase chain reaction of restriction fragment length polymorphism (PCR-RFLP) of intergenic spacer region (IGS) using 10 restriction enzymes, *Alu* I, *Bsu* 15I, *Bsu* RI, *Eco* 88I, *Eco* RI, *Hin* 6I, *Hin* fI, *Msp* I, *Rsa* I and *Xho* I was used to characterize the isolates of *Fusarium*. The restriction patterns produced showed highly variable patterns and from UPGMA cluster analysis showed that generally most of the isolates of *Fusarium* from the same species were clustered in the same cluster. In mating studies, only 60.5% of the isolates were successfully crossed-fertile with at least one of five mating populations, A (*G. moniliformis*), B (*G. sacchari*), C (*G. fujikuroi*), D (*G. intermedia*) and E (*G.*

subglutinans). From phylogenetic analysis of translation elongation factor-1 alpha gene (TEF-1α), six species of *Fusarium*, *F. andiyazi*, *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. sacchari* and *F. verticillioides* were identified. The topologies of the phylogenetic trees generated using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) were almost similar in which isolates with the same identity were clustered in a same clade. Generally, the identity of *Fusarium* isolates section Liseola can only be confirmed by using sequences of TEF-1α gene, and the results from morphological characteristics and mating studies can be used as additional information to assist in identification and characterization of *Fusarium* species section Liseola.

CHAPTER ONE

INTRODUCTION

The agriculture industry in Malaysia is growing rapidly due to increasing of population and high demands of raw materials for food processing industry. In the Ninth Malaysia Plan, parts of Malaysian government's aims are to increase the food self-sufficiency and revitalize agricultural production which also are the factors to increase economic growth. Furthermore, several crops have been given emphasis in the Third National Agricultural Policy (1998-2010) in which among the objectives of the policy are to increase productivity, enhance competitive environment, encourage co-operation between agricultural sector with other sectors and to utilize agricultural resources with sustainable basis. Among these crops, paddy (*Oryza sativa*), sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) are included as the important cash crops planted in Malaysia.

Paddy is the staple food of Malaysian and mainly planted for domestic consumption. With encouragement and subsidies such as minimum domestic price and fertilizer by Malaysian government given to paddy farmers, the paddy production has increased from 2,375,604 Mt in 2007 to 2,384,143 Mt in 2008 (Department of Agriculture, 2009). However, production of paddy was not enough and can only provide 60-65% of domestic consumption (Ibrahim and Mook, 2008).

Sugarcane in Malaysia is often associated with food processing industry and is planted in large scale for commercial production (Tan, 1989). Sugarcane cultivation in Malaysia is small compared to paddy but with improved variety, the yield has increased steadily although the sugarcane cultivation area has slightly decreased. Although the cultivated area of sugarcane has decreased to 14,670 Ha (2008) from 14,681 Ha (2006), the production of sugarcane has increased to 733,500 Mt in 2008 from 730,754 Mt in 2006 (Department of Agriculture, 2009).

Maize is planted as cash crop to generate extra income for farmers. Maize is served as human food, animal feed and industrial uses (Farnham *et al*., 2003). In Malaysia, the importation of maize has grown rapidly to US$398 million in 2006 from US$250 million in 2001 because local maize production is not enough to support the demands as maize is planted on a small scale. The cultivated area and production of maize were only 6,689 Ha and 37,458 Mt in 2008 respectively which cannot fulfill the domestic's demand (Warr *et al*., 2008; Department of Agriculture, 2009).

Like any other agricultural crops, paddy, sugarcane and maize are also infected by several types of diseases caused by plant pathogenic fungi. Among the plant pathogenic fungi, *Fusarium* spp. are often associated with diseases on paddy, sugarcane and maize. The most common *Fusarium* spp. associated with these three crops were species from section Liseola although other species have also been reported (Booth, 1971). Diseases caused by *Fusarium* spp. can reduce the yield and quality of the crops which lead to heavy losses to the farmers.

In Malaysia, *F. fujikuroi* is commonly associated with bakanae disease of paddy. Besides *F. fujikuroi*, other *Fusarium* spp. namely *F. proliferatum* and *F. verticillioides* are also involved in causing bakanae disease. Thus, there are more than one species of *Fusarium* section Liseola may infect paddy and cause the disease (Desjardins *et al*., 2007). Bakanae disease had caused approximately 50% losses in the yield of paddy in Asia (Webster and Gunnell, 1992), hence early detection are needed to prevent an outbreak of the disease which may lead to shortage of paddy.

In sugarcane, *Fusarium* spp. is often associated with stem rot and pokkah boeng disease which is one of the most common diseases infecting sugarcane in most plantations in Asia. In Malaysia, *F. moniliforme* var. *subglutinans* has been reported as the causal pathogen of pokkah boeng disease (Geh, 1973) while Leslie *et al*. (2005) reported that *F. sacchari* is the casual agent of pokkah boeng disease in Asia. When infected by the disease, the yield can be reduced approximately 40.8-64.5%, depending on the cultivars that are planted (Dohare *et al*., 2003).

Several *Fusarium* spp. have been reported to cause ear rot disease as well as seed rot, root rot, seedling blight and stalk rot of maize. Approximately 10-30% of losses in production of maize worldwide are due to stalk rot disease. Besides that, fumoninsins, a mycotoxin produced by *Fusarium* spp. can cause as much as 90% losses of maize affected by Fusarium ear rot (Agrios, 2005). The most common species associated with these diseases are *F. proliferatum*, *F. subglutinans* and *F. verticillioides* which are among the *Fusarium* spp. in the section Liseola (White, 1999).

Most of *Fusarium* spp. associated with diseases on paddy, sugarcane and maize are members of section Liseola which has been reported to be morphologically similar and phylogenetically closely related (Nirenberg and O'Donnell, 1998). The similarities of morphological features are often seen in the shape of macroconidia and microconidia while the DNA sequences data of *Fusarium* spp. section Liseola often showed high similarities (O'Donnell *et al*., 2000b; Leslie and Summerell, 2006). Therefore, by using only one approach for identification and characterization of *Fusarium* spp. section Liseola is not sufficient.

Identification and characterization of *Fusarium* spp. are mainly based on the differences in anamorphic morphological features such as shapes of macroconidia and microconidia, presence and absence of chlamydospores, growth rate and pigmentation (Leslie and Summerell, 2006). However, *Fusarium* spp. have the ability to change morphologically and physiologically in order to survive in a new environment which caused problems and confusion for correct identification (Booth, 1971). Although *Fusarium* spp. section Liseola are difficult to identify using morphological characteristics, it is necessary as it allows the sorting of species before applying other method of identification and characterization (Leslie and Summerell, 2006).

When the morphological characteristics are not sufficient to identify the anamorph states, the teleomorph of *Fusarium* spp. are used to identify the mating populations which represent different biological species (Mansuetus *et al*., 1997). In this approach, sexually fertile members of the same mating population or species will produce perithecia with eight ascospores or fertile progeny. Several teleomorph

genera have been reported to be associated with *Fusarium* spp. and the most common teleomorph for *Fusarium* species in section Liseola is *Gibberella* (Booth, 1971). A total of ten mating populations were recognized and designated as mating population A to mating population J which were included in the *G. fujikuroi* species complex (Klaasen and Nelson, 1996; Klittich *et al*., 1997; Samuels *et al*., 2001; Britz *et al*., 2002; Seifert *et al*., 2003; Zeller *et al*., 2003; Geiser *et al*., 2005; Lepoint *et al*., 2005; Leslie *et al*., 2005).

To further characterize the *Fusarium* spp. section Liseola a combination of molecular characterization using polymerase chain reaction based technique (PCR-based technique) and phylogenetic analyses are commonly used. Among the PCR-based techniques, combination of PCR and restriction fragment length polymorphism (PCR-RFLP) is widely applied to observe the genetic variability among the *Fusarium* spp.. Various regions of ribosomal DNA (rDNA) such as 28S, 18S, 5.8S, intergenic spacer region (IGS) and internal transcribed spacer region (ITS) have been used in PCR-RFLP analysis. However, IGS region is commonly used in characterization of *Fusarium* spp. section Liseola because the region showed higher variability at intraspecific level and due to lack of selective constraints in IGS region, and it evolved faster than any other regions of rDNA (Donaldson *et al*., 1995; Appel and Gordon, 1996; Edel *et al*., 1997).

Phylogenetic analysis is applied in this study to confirm the identity of morphologically identified *Fusarium* spp. where isolates that were grouped in a smallest diagnosable clade are identified as the same species. Furthermore, the relationships or relatedness among closely related species such as *Fusarium* spp.

section Liseola can be determined from the phylogenetic analysis (Guadet *et al*., 1989; O'Donnell *et al*., 1998a). In phylogenetic studies of *Fusarium* spp., several genes and regions such as histone gene, translation elongation factor-1 alpha gene (TEF-1α), beta-tubulin gene, calmodulin gene, mitochondrial small subunit rDNA (mtSSU), 28S rDNA, mating-type genes (MAT) and ITS region have been sequenced and the relationship among closely related species have been determined (Steenkamp *et al*., 1999; O'Donnell, 2000; O'Donnell *et al*., 2000a; Aoki *et al*., 2001; O'Donnell *et al*., 2004). Among the genes and regions, TEF-1α gene which is a protein coding gene is commonly used in phylogenetic of *Fusarium* spp. as this gene is highly informative among closely related taxa, exist as single copy and non-orthologous which can provide useful information at species level (Geiser *et al*., 2004).

Integration of morphological, mating studies and phylogenetic approaches are essential for precise identification of *Fusarium* spp. section Liseola as well as to resolve taxonomic confusion among the isolates. Therefore, the objectives of the present study were:

- To isolate and identify isolates of *Fusarium* using morphological characteristics from paddy, sugarcane and maize.
- To determine the mating population of *Fusarium* spp. in section Liseola from the three hosts.
- To characterize and to observe the genetic variability of the *Fusarium* spp. section Liseola from the three different hosts using PCR-RFLP of IGS region.
- To reconfirm and to determine the phylogenetic relationship of *Fusarium* spp. section Liseola by using DNA sequences of TEF-1α gene.

CHAPTER TWO

LITERATURE REVIEW

2.1 Agriculture crops in Malaysia

Agriculture crops in Malaysia are categorized into three types namely, cash crop, industrial crop and subsistence crop. Cash crop are plants that grow for direct sale in domestic market or for exportation rather than use as personal food or feeding livestock and also provide profits from an off-farm source. The types of cash crop in tropical and subtropical countries are almost similar but are different from temperate countries due to differences in many factors such as climate, food preference and culture. For example, paddy, cocoa, sugarcane, bananas and coffee are common cash crops in tropical and subtropical countries (Fafchamps, 1992; Wikipedia, 2009).

In Malaysia, major cash crops are paddy, fruits, ornamentals and vegetables although agriculture sector is dominated by industrial crops such as oil palm, rubber and forestry products. According to the statistics provided by Department of Agriculture (Table 2.1), paddy is one of the most important cash crops compared to sugarcane and maize. The cultivated area of paddy has decreased from 676,111 Ha (2007) to 670,524 Ha (2008) but the production has increased from 2,375,604 Mt (2007) to 2,384,143 Mt (2008). Sugarcane cultivated area and production remains the same from 2007 to 2008 with 14,670 Ha and 733,500 Mt, respectively. The cultivated area and production of maize has increased steadily from 6,625 Ha and 36,438 Mt in 2007 to 6,689 Ha and 37,458 Mt in 2008.

Table 2.1: Cultivated area and production of major cash crop in 2007 and 2008 (Department of Agriculture, 2009)

Crop	2007		2008	
	Area (Ha)	**Production (Mt)**	**Area (Ha)**	**Production (Mt)**
Paddy	676,111	2,375,604	670,524	2,384,143
Fruits	287,327	1,871,262	290,561	1,886,680
Vegetables	42,832	694,811	44,974	816,244
Sugarcane	14,670	733,500	14,670	733,500
Maize	6,625	36,438	6,689	37,458
Flowers	1,895	154,974,350	1,965	16,595,290

Paddy, sugarcane and maize are affected by a large number of fungal diseases which are caused by pathogenic fungi. Infection by plant pathogenic fungi could damage the crop as well as reduced the yield and quality of the crop. One of the most common plant pathogenic fungi infecting agricultural crops are the species from the genus *Fusarium*. *Fusarium* spp. commonly caused vascular wilt of vegetables, flowers and plantation crops such as sugarcane and banana; root and stem rot diseases of maize and paddy (Agrios, 2005).

2.1.1 Paddy and diseases

Paddy (*Oryza sativa*) from the Poaceae family is cultivated mainly in tropical regions and approximately 90% of the total paddy field worldwide is found in Asia which reflected that rice is the main staple food in most Asian countries. Staple food is a food that provides calories and carbohydrate which usually served as part of every meal (Chin and Supaad, 1986; Jacquot *et al*., 2001). In Malaysia, paddy is the most important food product for consumption which provides approximately one-third of daily calorie intake (Chin and Supaad, 1986).

In Malaysia, at least 50 diseases have been reported to infect different parts of paddy plant from seedlings to mature plant. Among the diseases, ten diseases are considered as major which can cause severe reduction of paddy yield. The diseases are bacterial blight, blast, tungro, sheath blight, stem rot, leaf sheaths, false smut, foot rot, bacterial leaf streak and bakanae. Another 40 diseases such as leaf scald, sheath blotch, sheath spot, sheath rot, ragged stunt, kernel smut and others caused only minor injury on paddy but have the potential becoming more severe if the environmental conditions are favorable (Chin and Supaad, 1986).

One of the most well-known diseases caused by *Fusarium* is bakanae disease. Bakanae means foolish or bad seedling in Japanese. The disease was first described by Hori in 1898 and *F. heterosporum* was identified as the casual agent of the disease. Sawada (1918) found the teleomorphic stage of the *Fusarium* sp. in which the asci was visible on the disease plant but named the fungi as *Lisea fujikuroi* (cited from Sun and Snyder, 1981). In 1931, a study on bakanae disease conducted by Ito and Kimura, identified *Gibberella fujikuroi* as the causal agent and the anamorphic stage was called *F. moniliforme* (Sun and Snyder, 1981).

The first bakanae disease observed in Malaysia was in Kedah, Kelantan and Perak in 1985 during the second rice season and paddy cultivars, Seberang (MR 77) was found to be susceptible to *Fusarium* infection (Saad, 1986). Typical symptoms of bakanae disease are abnormal elongation of stem with yellowish-green in colour, it also produce adventitious roots on upper nodes and a layer of white to pink fungal mycelium grows and spreads over the lower part of the stems (Ou, 1987; Sun and Snyder, 1981).

Other paddy diseases caused by species of *Fusarium* are seedling blight in which the seed are rotted, lesions on the roots and coleoptiles, yellowing of leaves and the germination of seed is reduced; scab where lesions are found on the glumes, discoloration of the grain from white to yellow, salmon or carmine, the nodes become black in colour and disintegrate; stem wilting whereby the infected rice become light, shrunken and brittle, and the seeds will not germinate (Kasai, 1923; Ou, 1987).

2.1.2 Sugarcane and diseases

Sugarcane (*Saccharum officinarum*) is one of the most valuable economic plants from the grass family, Gramineae. Sugarcane is the main material of sugar industry and is recognized as an excellent crop for producing sugar (Williams, 1975). Various by-products after sucrose extraction have many other uses (Feldmann *et al.*, 2001). For example, bagasse (fibrous residue) is used as fuel in mills, paper products, fiber board, and wall board while filter mud are reused as manure in sugarcane fields. Molasses which high in carbohydrate contents are used as livestock feed (Purseglove, 1979; Paturau, 1982; Feldmann *et al.*, 2001).

Sugarcane is cultivated in tropical and subtropical regions in which the biggest producers are India, Brazil and China. The cultivation area of sugarcane in Malaysia is small which approximately 14,500 Ha for the last five years (2004-2008) and the sugarcane varieties for industrial production are Ragnar, F148 and F172 (Tan, 1989). There are two main sugarcane plantation company in Malaysia namely, Padang

Terap Plantation in Kedah and Kilang Gula Felda in Perlis. The production of sugarcane is decreasing in which the production was 951,772 Mt in 2003, but in 2008, it was 733,500 Mt. Therefore, Malaysia had to import sugar from other countries to fulfill the demands for local consumption (Warr *et al.*, 2008).

Sugarcane plant is susceptible to several types of diseases as well as pest infestations. Several pathogenic fungi, bacteria and viruses have been reported to cause diseases on sugarcane. Among the diseases caused by fungi are pineapple disease, pokkah boeng, red rot, rust, smut, stem rot, downy mildew disease and *Sclerophthora* disease. Diseases caused by bacteria and virus are gumming disease, leaf scald, ratoon stunting disease, mosaic, Fiji disease and yellow leaf syndrome (Williams, 1975).

One of the well known sugarcane diseases occurring in Malaysia is pokkah boeng caused by species of *Fusarium* which was originally described by Wakker and Went in 1896 in Java. The first pokkah boeng disease in Malaysia was reported by Geh in 1973. Pokkah boeng is referred to as malformed or distorted leaves. The common symptoms are chlorosis on the underside of the leaf, the base is narrower than normal leaf and sometime has irregular dark red spots. Serious infection can cause necrosis on the apical leaf which becomes wrinkled and shorter, formations of lesions on sheath and knife cuts sign on the stem (Ploetz, 2006). The level of severity varies depending on the sugarcane cultivar especially commercial varieties namely, Loethers, Striped preanger and Black Cheribon as well as the effect of environmental conditions. Pokkah boeng become a major sugarcane disease in almost all sugarcane plantations in which the crop is grown for commercial or industrial uses. For example, POJ 2878 is one of the sugarcane varieties that were widely grown in Java,

however, the variety was susceptible to pokkah boeng disease and caused severe damage to the sugarcane (Martin *et al*., 1961).

Other sugarcane diseases caused by *Fusarium* spp. are stem rot and wilt disease. Stem rot is caused by *F. moniliforme* where the parenchyma cells are discoloured to purplish-red, young roots become red in colour and turn purplish then soon decayed while the infected plant fail to develop any roots. Sugarcane wilt disease is caused by *F. moniliforme* and *F. sacchari* where the leaves gradually become yellowish and dry, eventually the whole plant dry and die while the inner tissues of the basal internodes have visible brick red colour (Martin *et al*., 1961).

2.1.3 Maize and diseases

Maize is a monoecious plant and herbaceous annual cereal from the Poaceae family. The plant is cultivated as source of raw food and food products for human, livestock feeds and the by-products are used for industrial purposes to produce paper goods, ceramics, textiles, industrial alcohols, chemical products and pharmaceuticals. Maize is the third most important food crop used directly or indirectly for human consumption compared to wheat and paddy (Marchand *et al*., 2001).

Maize is widely cultivated (over 150 million hectares) throughout the world where United States produced almost half of the world's harvest followed by China and Brazil. The world's total maize production was about 784 million tonnes in 2007 which was just slightly more than rice which was approximately 650 million tonnes and wheat, approximately 600 million tonnes (Food and Agriculture Organization of

the United Nations (FAO), 2009). A remarkable increase of maize production in Asia especially China and India was due to increase of maize-cropping area to meet the livestock feed requirements and also for direct human consumption (Marchand *et al*., 2001). The production of maize in Malaysia is relatively small but is increasing steadily from 33,483 Mt in 2006 to 37,458 Mt in 2008 (Department of Agriculture, 2009).

Maize planted in tropical region is more prone to disease infection compared to maize planted in temperate region. Tropical climate with hot and humid weather help to spread the pathogens and once infected the disease can spread rapidly (Wellman, 1972; Paliwal *et al*., 2000). Some maize diseases caused by fungi occurs world-wide such as leaf blights, leaf rusts, leaf spots, stalk rots and ear rots whereby seed rot, seedling blight, root rots, stalk rots, ear rots and downy mildew are caused by fungi and commonly found in tropical countries (Paliwal *et al*., 2000).

One of the most common diseases of maize is ear or kernel rot caused by several species of *Fusarium* namely, *F. verticillioide*s, *F. proliferatum* and *F. subglutinans.* The disease normally occurs on kernel randomly or in groups of kernel as well as on injured kernels (Millers, 1994). Infected maize has cottony whitish to pink mould on the ear or scattered throughout; and often brown in colour or has white streaks (Paliwal *et al*., 2000).

Fusarium verticillioides (syn. *F. moniliforme*) is considered as one of the major causal agents of maize stalk rots (De Leon and Pandey, 1989; Drepper and Renfro, 1990). The rot started at the roots until the lower internodes and gradually worsening

as the maize plant grow. Other symptoms are premature ripening of the stalk, stem cracking and easily fall over when touched (Paliwal *et al*., 2000).

Other maize diseases caused by species of *Fusarium* are root rot, seed rot and seedling blight. In root rot disease, the root of maize becomes weak, water-soaked and browning and the rot may spread to the main roots, seedling and crown tissue. Symptoms of seed rot and seedling blight of maize are the seeds decay and seedlings may die before growing out from the soil, rotting of roots and stems of seedlings at the soil line and the leaves become yellowish, followed by wilting and damping-off of the seedlings (Paliwal *et al*., 2000).

2.2 Mycotoxin production by *Fusarium* species

Species of *Fusarium* are well-known mycotoxin producer. Mycotoxin is a toxigenic secondary metabolite produced by fungi to colonize crops or harvested crops for survival. A wide range of mycotoxins are produced by *Fusarium* spp. such as fumonisins, trichothecenes, zearalenone, moniliformins and gibberellins (Flannigan, 1991). The occurrence of mycotoxin is often associated with plant diseases, contamination of feeds and foods that subsequently caused diseases to human and livestock (Marasas *et al*., 1984; Nelson *et al.,* 1994).

Gibberellins are produced by *Gibberella fujikuroi* involved in the infection of bakanae disease on paddy (Yabuta *et al*., 1934), stalk rot of maize and sorghum (Klittich and Leslie 1989) and pitch canker disease on pine tree (Correll *et al*., 1992). Some members of gibberellins are growth hormone in higher plants which promote

seed germination, stem elongation, fruit growth and induction of flowering. Abnormal growth occurred on the plant if excessive gibberellins accumulated in the plant (Bruckner *et al*., 1989; Hooley, 1994).

Fumonisins are the major mycotoxins produced by the *G. fujikuroi* species complex and some of the anamorphs are members of section Liseola such as *F. verticillioides*, *F. proliferatum*, *F. sacchari, F. fujikuroi, F. subglutinans, F. thapsinum, F. anthophilum* and *F. globosum* (Leslie *et al.*, 1992; Nelson *et al*., 1992; Sydenham *et al.,* 1997; Moretti *et al*., 1995; Leslie *et al.,* 1996; Desjardins and Plattner, 2000). A total of four series of fumonisins have been described, A, B, C and P. Among these four series, fumonisin B series (FB) is the most active fumonisins which have been found in maize, maize-based food and rice and associated with a number of diseases in both humans and livestock such as human esophageal cancer, leukoencephalomalacia in horse, pulmonary edema and hepatic syndrome in swine (Marasas, 1995; Ross *et al*., 1990; Harrison *et al*., 1990; Logrieco *et al*., 2002).

Moniliformin (MON) has been detected in maize, rice and tobacco and produced by *F. subglutinans, F. proliferatum* and *F. fujikuroi* (Marasas *et al*., 1986; Desjardins *et al*., 2000). Toxigenic MON in livestock's feed has caused several diseases in chicken, duckling and mouse such as muscular weakness, respiratory distress and death (Springer *et al*., 1974).

Trichothecenes has also been detected in maize, rice and other cereal grains, produced by various *Fusarium* spp. namely, *F. moniliforme, F. poae, F. equiseti* and *F. graminearum* (Logrieco *et al*., 2002). Two types of *Fusarium* trichothecenes have

been described, type A-trichothecenes and type B-trichothecenes. The immunosuppressive effect caused by trichothecenes has caused mycotoxicoses in livestock and human such as nervous disorder, hemorrhagic and emetic syndromes (Ueno *et al*., 1973; Ghosal *et al*., 1978; Logrieco *et al*., 2002).

Zearalenone (ZEA) is commonly produced by *F. moniliformin, F. semitectum, F. graminearum, F. equiseti, F. culmorum* and *F. cerealis*. It is one of the most widely distributed *Fusarium* mycotoxin in agricultural commodities such as maize and rice and is often associated with breeding problems in livestock and hyper-estrogenism in swine (Ozegovic and Vukovic, 1972; Kuiper-Goodman *et al*., 1987; Logrieco *et al*., 2002).

Beauvericin had been detected in contaminated maize and asparagus. In *Fusarium* spp. section Liseola, beauvericin is produced in large amount by *F. subglutinans* and *F. proliferatum* while *F. verticillioides* produced little amount of beauvericin (Logrieco *et al*., 1998).

A large number of mycotoxins are produced by *Fusarium* spp. which not only found in living plants or in harvested crops, but the interaction of mycotoxins and plants has caused changes in plant development and growth such as leaf necrosis and reduction of seed and root quantity (Desjardins *et al*., 1995; Desjardins and Plattner, 2000). As the results, the health of animals and human will be affected after consumption of contaminated food with mycotoxins (D'Mello *et al*., 1997; Desjardins, 2006).

2.3 History of *Fusarium* taxonomy

The genus *Fusarium* was first described by Link (1809) based on an asexual non-septa spore which was fusiform or canoe or banana-shaped, borne on a stroma and was based on *F. roseum* (Booth, 1971). After Link, a large number of *Fusarium* species were described, however, most of them were poorly defined and the type specimens were no longer available. With numerous proposed characteristics, varieties, forms and various types of culture media used in identification and characterization had made the identification system of the genus *Fusarium* become complicated. This complicated system was then simplified by Wollenweber and Reinking as they reformulated the species concept of *Fusarium* species (Leslie and Summerell, 2006).

In 1935, an intensive study and simplified classification system of *Fusarium* spp. was developed by Wollenweber and Reinking with approximately 1,000 species. In Wollenweber and Reinking system, the species with common similarities were grouped into the same sections based on the shape of the foot or basal cells of macroconidia; presence and the shape of the microconidia; occurrence and location of the chlamydospores. Sixteen sections were described namely, Arachnites, Arthrosporiella, Discolor, Elegans, Eupionnotes, Gibbosum, Lateritium, Liseola, Macroconi, Martiella, Pseudomicrocera, Roseum, Spicarioides, Sporotrichiella, Submicrocera and Ventricosum, with 65 species, 55 varieties and 22 forms. Description and separation of species were based on colour of stroma; present and absent of sclerotia; length, width and the number of septa in the macroconidia. Species identification was based on culture variations. However, cultural media and incubation periods were not standardized and the culture used was not originated

from single conidia. The classification system by Wollenweber and Reinking (1935) was complex and difficult, and was mainly based on the differences showed by the isolates rather than the similarities of the isolates.

In the 1940s and 1950s, Snyder and Hansen (1954) carried out a comprehensive study on the taxonomy and biology of *Fusarium* spp. They initiated the use of single spore technique and focused on similarities rather than the differences between isolates. Snyder and Hansen reduced the number of *Fusarium* spp. from thousand to just nine species namely, *F. episphaeria, F. lateritium, F. moniliforme, F. nivale, F. oxysporum, F. rigidiuscula, F. roseum, F. solani* and *F. tricinctum* based on the morphological features of macroconidia and the variability of the *Fusarium* spp. Part of Snyder and Hansen's description of *F. oxysporum* and *F. solani* are widely accepted. However, the lumping of several sections from Arthrosporiella, Discolor, Gibbosum and Roseum into only one species, *F. roseum*, has caused a lot of confusion and disagreement among taxonomists working on *Fusarium*.

In the mid-1930s, Raillo developed a classification system based on the cultures from single conidia but it was only published in 1950. Raillo concluded that the shapes of apical cell was the main character for species determination while incurvature and width of conidia, length of apical cell and number of septa were used to separate subspecies and varieties. Furthermore, the cultural characters such as pigmentation, mode of spore formation and presence of sclerotia were used to separate special forms (Nelson *et al*., 1983).

Bilai (1955) introduced a classification system which included nine sections, 26 species and 29 varieties and combining section Liseola with section Elegans and section Gibbosum with section Discolor. The system was based on cultural variability and focused on the effects of temperature, moisture and media composition (Nelson *et al*., 1983).

Booth (1971) published a classification of *Fusarium* spp. based on the primary features of macroconidia and conidiogenous cell bearing microconidia while growth rate as secondary feature. Booth included the information on the teleomorph stage (perfect stage) that was related to some of the *Fusarium* spp. and included keys to characterize the isolate into sections and species which made the identification much easier especially to mycologists, plant pathologists and other researchers who work with *Fusarium* spp.

In 1986, Joffe published a classification system based on the combination of sections described by Wollenweber and Reinking and some species described by Gerlach. Joffe studied and examined a large quantity of *Fusarium* spp. from diseased plants and soil which were collected from warm, semiarid climate (Isreal) and cool climate (Russia). Based on the findings, 13 sections, 33 species and 14 varieties were described.

The concept of Wollenweber and Reinking was continued by Gerlach and Nirenberg (1982) in which they described 78 species that were arranged in sections with clear photographs and line drawings to enhance the original drawings by Wollenweber and Reinking. Gerlach and Nirenberg used eight different media which were used by

Wollenweber and Reinking and emphasized on the differences of the isolates produced by a single culture on different media.

Nelson *et al*. (1983) identification and classification system is a combination of several *Fusarium* classification systems produced by other researchers and their own research. For identification purposes, the cultures were grown on standardize media and originated from single conidia. Their system included *F. oxysporum* and *F. solani* described by Snyder and Hansen (1954), formation of conidiophores described by Booth (1971) and toxigenic species in several sections proposed by Wollenweber and Reinking (1935). They had reduced the number of species and combining varieties and forms into appropriate species because most of the described varieties and forms may have been cultural variants. In Nelson *et al*. (1983) classification, the uniform morphological features of macroconidia, microconidia, conidiophores and chlamydospores are essential for characterization and identification of *Fusarium* spp.

Burgess *et al*. (1994) emphasized less on the use of sections for species identification because the boundaries to differentiate species are difficult especially when introducing new species. The manual by Burgess *et al*. (1994) was based on examination of more than 40,000 cultures of *Fusarium* spp.

Leslie and Summerell (2006) published a manual which contain a compilation of species descriptions by several researchers. The used of three species concepts namely morphological, biological and phylogenetic species concept were included to give overall view and guidance for species identification.

From the beginning, the genus *Fusarium* is usually described as an imperfect fungi in which the asexual spores are produced on or within aerial hyphae and has unknown or lack of sexual structures and reproduction cycle (Agrios, 2005). However, over the years, the classification of the genus *Fusarium* changes after numerous studies have been carried out. The latest classification of the genus *Fusarium* is as follows (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=5506), which can only be found in the website.

Superkingdom: Eukaryota

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Subclass: Hypocreomycetidae

Order: Hypocreales

Genus: *Fusarium*

Members that included in the subphylum, Pezizomycotina produced sexual and asexual spores (dominant) in their life cycle. The relationship between sexual (teleomorph) and asexual (anamorph) stage of *Fusarium* spp. in section Liseola is still uncertain as these two stages seldom occur at the same time and/or on the host. If *Fusarium* spp. in section Liseola have sexual stage, then it will be classified as *Gibberella* spp. in Nectriaceae (family) (Kirk *et al*., 2001).

Until today, the taxonomy of the genus *Fusarium* is still evolving. There are still a lot of *Fusarium* spp. that are not well-described especially species from tropical and subtropical plants due to lack of information on described symptoms on tropical crops as well as similarities and overlapping morphological features especially species in the section Liseola (Summerell *et al*., 2003).

2.4 Species concept for identification of *Fusarium* spp.

The conflicting classification systems suggested by different researchers has caused difficulties for the identification of *Fusarium* spp. (Edel *et al*., 1996; Mishra *et al.,* 2003). Nowadays, *Fusarium* spp. that are morphologically similar or sibling species can be differentiated and identified with combination of different methods by using morphological, biological and phylogenetic approaches. Although the answers from using these three approaches may be different or similar, it will somehow provide information on species identity as well as the evolution or phylogeny of the species (Leslie *et al*., 2001).

Based on Linnaean definition, morphological species concept is based on the morphological features showed by an individual that represent the variation within the whole species and distinguishable differences from other species (Mayr, 1963). According to Taylor *et al.* (2000), morphological species concept is applied widely because of its broad applicability to any fungal taxa and had been used more than hundred years for identification of fungal species.

For *Fusarium* spp. identification, morphological species concept is useful during preliminary characterization and classification while biological and phylogenetic species concepts are being applied when a new species is found or the readily usable morphological features used to describe a particular species were not sufficient to separate isolates at species level (Leslie and Summerell, 2006).

According to Mayr (1963), biological species means species as groups of populations that can or have the potential to interbreed with each other. For *Fusarium* spp., biological species are a category or group of species that are sharing or probably sharing a collective of genetic information that are available within the sexual reproductive population rather than sharing the same physical and physiological features (Leslie *et al.,* 2001) and involved interbreeding within the same group of species. If fertile progeny is produced as a result of the interbreeding, it will be assigned to a same species. Each of these groups is termed as mating population and is regarded as a distinct species (Leslie *et al*., 2007). Biological species concept using mating studies has been applied extensively in study of *F. solani* (*Nectria haematococca*) and within species of *Fusarium* in section Liseola (*G. fujikuroi* species complex) (Leslie and Summerell, 2006).

In phylogenetic species concept, a species can be differentiated from other species by using the smallest phylogenetic clade of individuals that shared the same set of characters (Nixon and Wheeler, 1990). When phylogenetic species concept is applied in *Fusarium* taxonomy, it gives additional information on evolutionary history of *Fusarium* spp. or relatedness between taxa. In practice, phylogenetic species concept usually employed molecular markers such as TEF-1α, beta-tubulin gene, IGS and

ITS regions of rDNA to evaluate the evolutionary relationships as well as to distinguish species in the same or closely related clade. From molecular data, several species of *Fusarium* have been described as different species whereby it was previously identified as the same species (Klittich *et al.*, 1997; Nirenberg and O'Donnell, 1998; Leslie *et al.,* 2001; Marasas *et al.,* 2001).

2.4.1 Identification based on morphological characteristics

Traditional taxonomic system for identification of *Fusarium* spp. was based on morphological characteristics which was the first step to identify and sorting of isolates in the genus. Morphological characteristics of *Fusarium* can be divided into primary and secondary characteristics. The primary characteristics used for identification are macroconidia, microconidia, chlamydospores and conidiophores. Pigmentation, colony morphology and growth rates are the secondary characteristics (Burgess *et al.,* 1994; Leslie and Summerell, 2006).

Macroconidia is the main feature for identification of *Fusarium* spp. The fusoid macroconidia with a foot-shaped cell together with the number of septate, shapes of apical and basal cell are the main characteristics for identification and classification. There are four general forms of apical cells namely, blunt, papillate, hooked and tapering and basal cell, foot, elongated foot, distinctly notched and barely notched cells (Burgess *et al*., 1994; Leslie and Summerell, 2006).