MORPHOLOGICAL CHARACTERISTICS, DISTRIBUTION, AND MYCOTOXIN PROFILES OF *Fusarium* SPECIES FROM SOILS IN PENINSULAR MALAYSIA

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%	percentage
&	and
®	Registered
°C	Degree of celcius
°F	Degree of Fahrenheit
µl	microliter
µl/g	Microliter per gram
µm	Micrometer
AFLP	Amplified Fragment Length Polymorphism
a_w	Water availability
BEA	Beauvericin
C	Carbon
Ca	Calcium
CFU	Colony formation unit
CLA	Carnation leaf agar
cm	Centimeter
Co	Cobalt
CO_2	Carbon dioxide
Cu	Copper
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EF-1α	α-elongation factor
f. sp.	formae speciales
FA	Fusaric Acid
Fe	Ferum
$FUMB_1$	Fumonisin B_1
g	gram
GA	Gibberellic Acid
GLC	Gas-Liquid Chromatography
H	Hydrogen
H_2O	Water
H_2SO_4	Sulfuric acid
HPLC	High Performance Liquid Chromatography
hrs	hours
K	Kalium
kg/cm^2	kilogram per centimeter square
M	Molarity
MBTH	Methylbenzhothiazolonehydrocholoride
Mg	Magnesium
mcf	Moisture correction factor
mg	milligram
min	minutes
ml	milliliter
ml/min	milliliter per minute
mm^2	millimeter square
Mn	Mangan
MON	Moniliformin

N	Nitrogen
$Na_2S_2O_3$	Sodium thiosulfate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
nm	nanometer
No.	Number
O	Oxygen
P	Phosphorus
p.s.i.	pounds per square inch
PDA	Potato Dextrose Agar
PPA	Pentachloronitrobenzene agar
ppm	parts per million
RAPD	Randomly Amplified Polymorphic DNA
R_f	Retention factor
RFLP	Restriction Fragment Length Polymorphism
S	Sulphur
SDS	Sudden Death Syndrome
SEA	Soil extract agar
sp.	Species
TLC	Thin layer chromatography
UV	Ultra violet
W	Watt
w/w	weight per weight
WA	Water agar
ZEN	Zearalenone
Zn	Zinc

CIRI MORFOLOGI, TABURAN DAN PROFIL MIKOTOKSIN DARIPADA SPESIES *Fusarium* DARIPADA TANAH DI SEMENANJUNG MALAYSIA

ABSTRAK

Fusarium merupakan salah satu genus kulat yang paling dikenali dan penting kerana kepelbagaian, kosmopolitan, dan keupayaannya sebagai penyebab kepada sebilangan penyakit yang parah terhadap tumbuhan, manusia, haiwan, dan juga mikotoksikosis. Spesies *Fusarium* biasanya dijumpai di dalam tanah di semua kawasan geografi utama dunia. Walau bagaimanapun, ramai penyelidik menemui kesukaran untuk mengenalpasti spesies *Fusarium* secara morfologi kerana banyaknya persamaan dan sifatnya yang berubah-ubah. Justeru itu, objektif utama kajian ini adalah untuk mengenalpasti spesies *Fusarium* yang telah dipencilkan daripada tanah di Semenanjung Malaysia dengan mengkaji ciri-ciri morfologi, taburan dan kepadatan, dan menyelidik profil mikotoksinnya. Daripada 55 sampel komposit tanah yang berbeza dari segi jenis penggunaan dan tanamannya, sebanyak 492 isolat *Fusarium* telah dikenalpasti dan dicamkan menjadi 10 spesies dan satu spesies yang tidak dapat dicamkan. Spesies yang paling dominan adalah *F. solani* (39%), diikuti oleh *F. oxysporum* (30%), *F. semitectum* (14%), *F. proliferatum* (7%), *F. subglutinans* (3%), *F. compactum* (2%), *F. equiseti* (2%), *F. chamydosporum* (1%), *F. merismoides* (1%), *F. dimerum* (0.8%), dan *Fusarium* sp. 1 (0.2%). Penggunaan ciri-ciri morfologi sebagai satu kaedah pengecaman adalah mudah malah pembezaan antara spesies-spesies juga dapat dilakukan. Justeru itu, kekunci pengecaman spesies *Fusarium* daripada tanah telah dibuat berdasarkan ciri-ciri morfologi

tersebut. Jenis penggunaan dan tanaman ke atas tanah serta sifat-sifat tanah memberi kesan kepada taburan dan populasi spesies *Fusarium*. Spesies *Fusarium* lebih padat di dalam tanah-tanah pertanian, diikuti oleh tanah yang berasid, berlom, dan berkelembapan tinggi. *F. solani* merupakan spesies yang paling lazim dijumpai iaitu 52 daripada 55 sampel tanah (94.5%). Di dalam kajian mengenai profil mikotoksin, perbezaan profil yang ditunjukkan oleh spesies-spesies tertentu dapat digunakan sebagai pengukuh kepada pengecaman spesies secara morfologi. Moniliformin, zearalenone (0.81 – 205.88 µl/g), dan beauvericin (0.94 – 2122.06 µl/g) telah dikesan dari sebanyak 24 daripada 28 isolat yang diuji. Fumonisin B_1 pula tidak dikesan di dalam mana-mana isolat yang diuji. Beberapa ekstrak mikotoksin adalah sangat toksik terhadap larva udang air masin iaitu moniliformin (100%), zearalenone (100%), dan beauvericin (98%). Keputusan kajian terhadap profil mikotoksin menunjukkan keupayaan spesies *Fusarium* tertentu di dalam penghasilan toksin boleh membantu mengukuhkan keputusan pengecaman secara morfologi dan dapat menilai potensi ketoksikan spesies *Fusarium* daripada tanah. Oleh itu, hasil daripada kajian-kajian ini memberikan maklumat terkini berkenaan taburan dan profil mikotoksin oleh spesies *Fusarium* yang telah dipencilkan daripada tanah di Semenanjung Malaysia.

MORPHOLOGICAL CHARACTERISTICS, DISTRIBUTION, AND MYCOTOXIN PROFILES OF *Fusarium* SPECIES FROM SOILS IN PENINSULAR MALAYSIA

ABSTRACT

Fusarium is considered as one of the most interesting and important group of fungi, because of the diversity, cosmopolitan, and ability to cause serious diseases on plants, humans, animals, as well as mycotoxicoses. *Fusarium* species is commonly found in the soils in all major geographic regions of the world. However, many researchers find it difficult to identify *Fusarium* into species level morphologically due to the close similarities and vast variabilities within the species. Hence, the main objectives of these studies were to identify *Fusarium* isolated from soils in Peninsular Malaysia into species by using morphological features, to study their distributions and density, and to investigate their mycotoxin profiles. From 55 composite soil samples with different vegetation and land use throughout Peninsular Malaysia, 492 isolates of *Fusarium* were identified into 10 species and one unidentified species. The most dominant species were *F. solani* (39%), followed by *F. oxysporum* (30%), *F. semitectum* (14%), *F. proliferatum* (7%), *F. subglutinans* (3%), *F. compactum* (2%), *F. equiseti* (2%), *F. chamydosporum* (1%), *F. merismoides* (1%), *F. dimerum* (0.8%), and *Fusarium* sp. 1 (0.2%). The identification by using morphological characteristics was convenient and able to distinguish the species. Thus, the key for identification of *Fusarium* species from soils was presented. Soil vegetation and usage as well as other soil characteristics have

an influence in the distribution and population of *Fusarium* species where *Fusarium* species are more abundant in cultivated, followed by acidic, loamy, and moist soils. *F. solani* was the most prevalent species, being presence in 52 out of 55 samples (94.5%). In the study of mycotoxin profiles, some species could be distinguished from others that could be used to complement the morphological species identification. Moniliformin, zearalenone (0.81 – 205.88 µl/g), and beauvericin (0.94 – 2122.06 µl/g) were detected from 24 out of 28 isolates tested. Fumonisin B_1 was not detected in any of the isolates. In addition, a few extract of mycotoxins were highly toxic to brine shrimp larvae i.e. moniliformin (100%), zearalenone (100%), and beauvericin (98%). The results showed the ability of certain *Fusarium* species to produce toxins which may assist in the morphological identification, and the potential toxicity of *Fusarium* species isolated from soils. Thus, the findings in these studies provided the latest report on the distribution and mycotoxin profiles of *Fusarium* species isolated from soils in Peninsular Malaysia.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Soil

In soil sciences, soil is define as a body of earth crust that formed from stone and pebbles by the interaction of weather, living organisms, topography, and time (Brady, 1974; Jusop, 1981). Soil is therefore a very important component that covered the earth crust. All living organisms rely on this important earthy component for shelters, foods, nutrients, and other purposes. The relationship between soils and living organisms has been very intimate and valuable. It is a natural base medium that contains variable of organisms, ions, and nutrients which is a suitable habitat for flora and fauna, especially for the microorganisms. The soil is therefore the home of innumerable forms of plants, animals, and microbial lifes.

1.2 Life In The Soil

Life in the soils is amazingly diverse, ranging from microscopic single-celled organisms to large burrowing animals. Every organisms lives on the surface or in the soils affects the chemical and physical properties of soils. The organisms can be considered as higher plants, vertebrates, microorganisms, and mesofauna. Higher plants contribute to the addition of organic matter or litter to the soil surface. The litters provide nutrients for the decomposers such as soil microorganisms. Plants extract water and nutrients from the body of the

soil and under natural conditions return most of the nutrients to the surface in the litters which decomposes and releases the nutrients, rendering them available for re-absorption. Mesofauna is a group of organisms that includes earthworms, nematodes, mites, springtails, millipedes, some gastropods and many insects, particularly termites. Similar to microorganisms, their distribution is determined almost entirely by their food supply and therefore their populations are concentrated in the top 2 to 5 cm; only a few, such as earthworms penetrate below 10 to 20 cm. The concentration of each organism varies greatly from place to place according to vegetation.

The distribution of microorganisms in soils is determined by the presence of suitable nutrients. Therefore, microorganisms occur in the greatest numbers in the surface horizon of the soils which is a teeming mass of biological activity. Microorganisms are divided into two groups, the heterotrophs and the autotrophs. The former, including most of the bacteria, actinomycetes, and fungi, obtain the nutrients and energy from plant and animal remains, while the latter derive their body carbon solely from the carbon dioxide of the atmosphere. Therefore, the heterotrophs are principally responsible for the decomposition of litters. Most microbes require an aerobic environment and have optimum temperature requirements of 25-30°C.

Microorganisms in soils are very important in providing plants with minerals (Gray & William, 1971). Furthermore, each microorganisms present in the soils have their own role. Bacteria, being the highest number of organisms within the top 15 cm of the soil, play an important role in gas cycles such as nitrogen, while fungi decaying organic substances that add cellulose and inorganic substances into the soils (Brady, 1974). Soil fungi are critical to soil

environment where most of them are able to live in acidic conditions (Dalal, 1998). However, there is a great variation of microorganisms according to the depth of the soils. In addition, microorganisms are believed to be competing with each other in the soils where the group that is dominant constitutes the largest population (Gray & William, 1971). Norsiah (1990) reported that fungi are more dominant in acidic soils compared to other organisms.

1.3 Factors That Influence Microorganisms In Soil

The physical properties of soil include soil texture, structure, density, porosity, color, aeration, and water availability (a_w). These physical characteristics influence the water and air movements within the soils. In all of the physical properties, soil texture is the most important as it provides the ability to hold ions and nutrients, thus very important in soil classification. Furthermore, it influences the physical, chemical, and biological properties in soils. Hence, the soil microbes will definitely be affected by the type of soil properties. The texture of soils on the other hand, is determined by distribution of soil particle sizes i.e. sand, silt, and clay.

As we all know, water makes life possible to human beings as well as other living organisms on earth. So, the water content in soil is an important property for the survival of microbes. It regulates the climate of soil environment, dissolving soil minerals, and controls the amount of oxygen and other gases in the soil. These, in turn, will affect the density and diversity of microbes in the soils.

Chemical properties such as pH, base and mineral availability in soils also influences the microorganisms. Nutrient availability depends on pH conditions in the soils. When the pH value increases, the availability of ferum (Fe), mangan (Mn), zinc (Zn), copper (Cu) and cobalt (Co) will decrease. Microorganisms are most abundant in soils with neutral pH range.

1.4 Soils In Malaysia

The pH values of most soils in Malaysia are from 4.5 to 5.5. Malaysia does not experience an extreme high and low temperatures. The average of minimum temperature is 23.3°C (74°F) and maximum temperature is 30.5°C (87°F) (Jusop, 1981). However, the average daily temperature taken in 2007 is between 22°C and 28.1°C. The highest temperature recorded in Malaysia was 40.1°C on April 9th, 1998 in Chuping, Perlis (www.met.gov.my). However, there are no significant differences in soil temperatures around Malaysia. The soil moisture content in Malaysia is generally at 60 – 70% for the whole year (Figure 1.1).

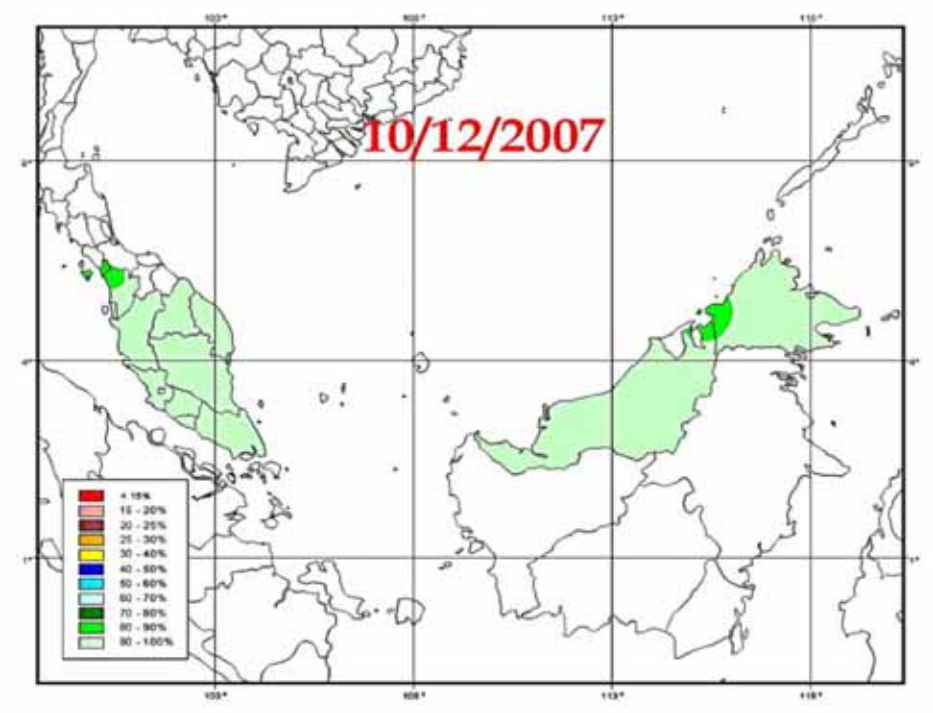

Figure 1.1. Distribution of soil moisture content in Malaysia (www.met.gov.my) in December 2007

1.5 The Genus of *Fusarium*

The genus of *Fusarium* has been considered as one of the very interesting and important group of fungi, because of its diversity, cosmopolitan, and responsible for numerous plant diseases, storage rots, and human as well as animal toxicoses and mycoses (Nelson *et al*., 1981; Liddell, 1991; Nelson *et al.*, 1994; Summerell *et al.*, 2003). These fungi are facultative parasites that live as parasites or saprophytes depending on their host. Furthermore, most *Fusarium* species could continue living in soils, or being parasites or saprophytes to grasses if no available host around. They produce dormant structures, mostly in the form of chlamydospores to keep on living in soils for many years before these structures are stimulated to grow. Apparently, these fungi are lack of sexual state, therefore, they are known as fungi imperfecti

(Fincham *et al.*, 1979). The identification and system of classification of *Fusarium* species are very complex. Although more than 80 species have been recognized, there is still a problem to identify *Fusarium* into species morphologically because of different classification systems used by researchers throughout the globe (Leslie & Summerell, 2006). However, morphological characteristics are still the most important criteria to identify *Fusarium* into species (Leslie *et al*., 2001).

As already known, most *Fusarium* species are pathogenic to plants. At least one *Fusarium*-associated disease is found on many plants (Leslie *et al*., 2006). The fungi have caused plant diseases such as crown rots, head blights, scabs, vascular wilts, root rots, and cankers. The most disastrous disease caused by *Fusarium* species in agricultural history throughout the world was the infection of *F. oxysporum* f. sp. *cubense* on banana in Panama, thus known as Panama disease (Ploetz, 1990) affecting the whole Panama's economic sectors in the agricultural industry. Another major event caused by this genus was the disease called *Fusarium* head scab on wheat and barley in the United States (Windels, 2000). In Southeast Asia, Asia Pacific, and Australia, Panama disease caused serious losses to the banana plantation and industry (Chris *et al*., 2000; Hwang & Ko, 2004). Besides Panama disease, there are some other *Fusarium*-associated diseases that give problems to agricultural industry such as pokkah-boeng on sugarcane, bakanae disease on rice, vascular wilts on oil palm, and asparagus decline (Salleh, 2007).

Fusarium species are also widely distributed in all major geographic regions of the world (Burgess, 1981; Nelson *et al*., 1994). They are commonly found in soils, and persist as chlamydospores or as hyphae in plant residues

and organic matter (Gordon, 1959; Booth, 1971; Burgess, 1981). However, many *Fusarium* species are abundant in fertile cultivated and rangeland soils, rather than in forest soils (Burgess *et al.,* 1975; Burgess *et al.*, 1988; Jeschke *et al.,* 1990). According to Nash & Snyder (1965), *Fusarium* colony was found abundant and diverse in cultivated soils. A high degree of variability in morphology and physiological characteristics enable some species such as *F. oxysporum* and *F. equiseti* to occupy the diverse ecological niches in many geographic regions (Burgess *et al.*, 1989). In Malaysian soils, an intensive study on diversity of *Fusarium* species was first conducted by Lim (1971). Because of its wide range distribution in soils, they are also known as soil-borne fungi.

1.6 Mycotoxins Produced by *Fusarium* species

Besides the diversity and distribution around the world, toxic substances produced by *Fusarium* species in post-harvest products are what matters most. *Fusarium* species produced a range of mycotoxins that could pose a serious threat to plant, animal and human healths (Marasas *et al.*, 1984; Joffe, 1986, Salleh & Strange, 1988; Salleh, 1998). Mycotoxins are secondary metabolites produced by fungi that are associated with a variety of animal disorders and some human health problems. Mycotoxicoses are diseases or disorders caused by the ingestion of foods or feeds made toxic by these fungal metabolites. Trichothecenes, zearalenone, and fumonisins, for instance, are the major *Fusarium* mycotoxins produced in infected maize kernels (D'Mello *et al*., 1999; Logrieco *et al.*, 2002). *F. verticillioides*, *F. proliferatum*, and *F. nygamai* produced mycotoxins called fumonisins (Thiel *et al*., 1991). These toxins could

cause oesophaegal cancer to humans and may cause allergic or carcinogenic symptoms, in long term consumption (Bottalico, 1998). Many mycotoxins produced by *Fusarium* species were discovered in cereals especially maize. For that discovery, the infected maize kernels are of great concern worldwide. Furthermore, it was estimated that 25% of the world food crops are affected by mycotoxins (Charmley *et al*., 1995). Mycotoxin profiles from *Fusarium* strains in temperate region have been studied very intensively which resulted a one-sided view of the ability of the strains from tropical region to produce mycotoxins. *Fusarium* mycotoxins were allegedly used as biological warfare agents in Asia. So, more studies on mycotoxin profiles was suggested by Salleh (1998) following the discovery of a new toxin, chlamydosporol from *F. chlamydosporum* isolated from rice in Penang (Savard *et al*., 1990).

In general, these studies were conducted to gain more information on some geographical factors on the diversity of *Fusarium* species in soils, morphological characteristics of the isolated species, and their potential in producing mycotoxins. Below are the listed objectives of the study:

1. To study the distribution and density of *Fusarium* species in soils of the Peninsular Malaysia.
2. To identify *Fusarium* species isolated from the soils by using morphological characteristics, and to determine the diversity of the species.
3. To investigate the mycotoxin profiles produced by *Fusarium* species isolated from the soils.

This study extends the previous research to the latest information on mycogeographical survey and diversity of *Fusarium* species in the soils of Peninsular Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Soils

2.1.1 Physical properties

Soils are classified into different textural groups according to the relative proportion of different sizes of mineral particles (Sharma, 2005; Coyne & Thompson, 2006). The mineral particles are clay, silt, and sand. There are 12 types of soil texture classified in the USDA soil texture triangle. Types of soil texture effects the soil physical, chemical, and biological properties (Coyne & Thompson, 2006). Some of the soil physical properties that were influenced by the texture are porosity, pore size distribution, water-holding capacity, and permeability. Furthermore, the texture influence the chemical properties or the nutrients in the soils i.e. P, K, Ca, organic matters and others (Table 2.1). A soil with high amount of clay particles has higher nutrient-holding capacity and greater organic matter content than sandy soils (Coyne & Thompson, 2006). Consequently, the availability of soil nutrients influences the presence of microorganisms. Moreover, microorganisms could attach to the large surface area of soil particles such as clay to colonize. Therefore, soil texture is an important factor that determines the presence and level of microbes.

Table 2.1: Separates of soil particle size associated with nutrient content (Coyne & Thompson, 2006)

Separate	Total P (%)	Total K (%)	Total Ca (%)
Sand	0.05	1.4	2.5
Silt	0.10	2.0	3.4
Clay	0.30	2.5	3.4

2.1.2 Vegetation

Vegetation refers to the plants found in a particular environment (Hornby, 1995). In the world, major types of world vegetations are tropical and subtropical forests, savannas, temperate grasslands, heath lands, deserts and desert-like shrubs, temperate forests, tropical alpines, marine and estuarine wetlands, and freshwater wetlands (Collinson, 1977). Climate is a major determinant of vegetation types (Brewer, 1994). Generally, major vegetation of Peninsular Malaysia is tropical rainforest. Tropical rainforest is the most complex biocoenosis life with a high order of dynamic organization and community interactions (Collinson, 1977). The annual precipitation in tropical rainforest is very high and the variation of temperature and humidity is very slight (Brewer, 1994). Furthermore, the soils in tropical rainforest are old, composed of aluminum and iron oxides, and acidic (Brewer, 1994). Eventually, forest can be divided into primary and secondary forests (Merrill, 1942; Numata *et al*, 2006). Primary forests comprised of a system with sufficient plant ages and minimal disturbances. The forests, therefore, are characterized by the presence of older trees, minimal signs of human disturbances, mixed-age stands, and presence of canopy openings. On the other hand, secondary forests comprised of woodland areas which have re-grown after a major disturbance such as fire, insect infestation, timber harvest, or wind throw, until a

long period of times has passed so that the effects of the disturbances are no longer evident (Corlett, 1994). The forests have only one canopy layer that allows sunlight to reach the forest floor, and colonized by pioneer species such as shrubs or jungles. Other vegetations can be grouped into types of plants or crops that cover the land i.e. perennial crops, annual crops, and grasslands. Perennial crops are plants that live for more than two years such as bananas, golden rods, mints, and dragon fruits. Furthermore, annual crops are groups of plants that usually germinate, flower and die in one year such as corns, lettuces, peas, cauliflowers, watermelons, beans, and rice. On the other hand, grasslands are areas where the vegetation is dominated by grasses and non-woody plants (Merrill, 1942; Collinson, 1977).

2.1.3 Nutrients

Nutrients in the soils can be divided into three groups i.e. basic nutrients, macronutrients, and micronutrients. Basic nutrients are composed of carbon (C), hydrogen (H), and oxygen (O). These basic nutrients come from water (H_2O) and carbon dioxide (CO_2). Plant parts that fell onto the soil are the source of these basic nutrients because of the structure of plants that are made of carbohydrates (starch, cellulose), hydrocarbons (fatty acids), and lignin (Coyne & Thompson, 2006). Macronutrients such as nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S) are available in the soils that are essential for plants. Moreover, micronutrients that are needed by plants such as iron (Fe), zinc (Zn), and others also available. In conjunction, the fertility of the soils is based on the availability of the nutrient. However, the

nutrients of soils depend on the types of soils and vegetations (Collinson, 1977; Coyne & Thompson, 2006).

2.2 Taxonomy of *Fusarium*

2.2.1 History of *Fusarium* classification system

The study of *Fusarium* taxonomy began on 1809 by a scientist named Link (Snyder & Toussoun, 1965). However, an intensive study about the classification system was done by Wollenweber and Reinking (1935) who introduced the use of sections in classifying *Fusarium* species into 16 sections (Appendix 1), 65 species, and 77 sub-specific varieties and forms (Appendix 2). Their taxonomic study was monumented in the publication of *Die Fusarien*. The monumental monograph becomes a standard reference in promoting *Fusarium* taxonomic systems afterwards (Nelson *et al.*, 1994). In the development of *Fusarium* taxonomical system, many researchers proposed their systems based on intensive studies on morphological characteristics. In general, the taxonomists were divided into two groups i.e. the lumpers and the splitters. Wollenweber and Reinking (1935), Raillo (1950), Bilai (1955), Gerlach and Nirenberg (1982),and Joffe (1986) are the group of splitters. They have separated the species into species, varieties, and forms. Gerlach and Nirenberg (1982), whom were the followers of Wollenweber & Reinking (1935), introduced 78 species in the genus. However, the species are determined by the differences not the similarities between each strain which leads to many new species or varieties. The philosophy of their system is difficult and complex (Nelson *et al.*, 1994). Following Gerlach and Nirenberg (1982), Raillo (1950)

and Bilai (1995) proposed their systems based on Wollenweber and Reinking (1935) in Russia. The systems were not well-understood where, for instance, they combined section *Liseola* with section *Elegans* and then combined section *Gibbosum* with *Discolor*. Another researcher the so-called in splitters group was Joffe (1986), who supposedly proposed a modern system but appeared to be a restatement of Wollenweber and Reinking's (1935) sections and species with some additions from Gerlach's species.

On the other hand, Snyder and Hansen (1940) began their studies of *Fusarium* taxonomy in 1930's and presented their results in 1940s. Snyder and Hansen (1940; 1941; 1945) are known as the ultimate lumpers as they compiled all the species from Wollenweber and Reinking (1935) into nine species. They combined sections *Arthrosporiella, Discolor, Gibbosum*, and *Roseum* into *F. roseum*. The lumping of the sections is confusing and not accepted by many *Fusarium* taxonomists. However, Snyder and Hansen (1940) are respected for their efforts on analyzing the species through single-conidium cultures. Their work on the variation of *F. oxysporum* and *F. solani* are well accepted among the taxonomists. The other taxonomists that are known as the lumpers are Messiaen and Cassini (1968), and Matuo (1972). Nelson (1991) stated that neither group (the splitters and the lumpers) produced a practical identification system for *Fusarium* species as the Wollenweber's system is too complex and the Snyder and Hansen's system is too simple.

Other than the splitters and the lumpers groups, there are groups of moderate taxonomists lead by Gordon (1944; 1952; 1954; 1956; 1960). Gordon's taxonomic system is closely related to Wollenweber and Reinking (1935), but he also considered Snyder and Hansen's system. Later, Booth

(1971) modified the Gordon system to the expansion of perfect stage information and the use of conidiophores and conidiogenous cells in his taxonomic system. He, successfully, separated the species in different sections based on the presence of monophialides and polyphialides. Then, Nelson *et al*. (1983) combined all the systems with their results to develop a practical approach in identification. Eventually, they reduced the number of species and combined the varieties and forms into appropriate species (Snyder & Toussoun, 1965; Nelson, 1991; Nelson *et al.*, 1983; Burgess *et al.*, 1994; Nelson *et al.*, 1994; Leslie & Summerell, 2006). The basic approach by Nelson *et al.* (1983) and Burgess *et al.* (1994) is accepted by many researchers. Recently, Leslie & Summerell (2006) published a *Fusarium* laboratory manual that unites all the taxonomical system with the latest techniques and methods for species identification. Furthermore, Leslie & Summerell (2006) integrates the morphological, biological, and phylogenetic species concepts. The difficulties and complexities of *Fusarium* taxonomical system is because of the connection of anamorph-teleomorph, section relationships, species delimitation, mutational variants, and subgroup identification (Windels, 1991). In addition, the wide range of scientists and technologist working with *Fusarium* species has created difficulties in international agreement of systematic *Fusarium* taxonomy (Liddell, 1991).

2.2.2 Primary characteristics

A systematic identification process is needed to identify the complexity of *Fusarium* taxonomy (Summerell *et al.*, 2003). Thus, a systematic approach that was introduced by Burgess *et al.* (1994) and Leslie & Summerell (2006) in their

manuals are helpful to identify *Fusarium* species morphologically. *Fusarium* species produced three types of spores i.e. microconidia, macroconidia, and chlamydospores (Nelson *et al*., 1994). However, the presence of macroconidia is the most important characteristic that distinguished *Fusarium* species from other genus.

Macroconidia are formed in sporodochium and had a shape of a moon crest or a boat or banana with multiseptum (Alexopoulus *et al*., 1996). Basically, there are three shapes of macroconidia i.e. straight or needle-like, dorsiventral curvature, and dorsal curvature. The shapes of the end, apical and basal cells are important characteristics to determine species. Generally, the apical cells have four shapes i.e. blunt, papillate, hooked and tapering, while the basal cell also with four shapes i.e. foot-shaped, elongated foot shape, distinctly notched and barely notched (Leslie & Summerell, 2006).

Microconidia are produced only at the aerial mycelium from conidiogenous cells not sporodochia. There are two types of conidiogenous cells i.e. monophialides and polyphialides. The former with only one single opening while the latter with two or more openings per cell (Alexopoulus *et al*., 1996; Leslie & Summerell, 2006). The arrangement of microconidia on the conidiogenous cells either in singly, false heads, or chains are important in identification. Moreover, the presence and absence of microconidial chain is very important to identify species in section *Liseola* (Hsieh *et al.*, 1979; Fisher *et al.*, 1983). Furthermore, the shapes of microconidia are oval, reniform, obovoid, pyriform, napiform, globose, and fusiform (Leslie & Summerell, 2006).

Another type of spore are chlamydospores that have a thick wall with a lipid substance inside that give the fungus the ability to survive in an extreme

condition even outside the host (Alexopoulus *et al.*, 1996). Some *Fusarium* species produced chlamydospores which become an important characteristic for identification. The formation of chlamydospores could be singly, doubly, clumps, and in chains (Leslie & Summerell, 2006). In the laboratory, the formation of chlamydospores takes a long time, sometime up to six weeks. The chlamydospores could be formed in the aerial mycelium or embedded on the agar (Nelson *et al.*, 1994). Furthermore, the chlamydospores germination is influenced by water content in the soils and root exudates (Cook & Flenttje, 1967).

The other important morphological characteristic is mesoconidia. Mesoconidia are the fusoid conidia that are longer than microconidia with 3-4 septa but shorter than macroconidia with lack of foot-shaped and notched basal cell (Leslie & Summerell, 2006). These conidia are produced in the aerial mycelium on the polyphialides that appear as “rabbit ears” when viewed *in-situ*. Furthermore, this type of conidia is the most important feature to distinguished F. *semitectum* (Leslie & Summerell, 2006). These morphological features of *Fusarium* species especially in section *Elegans* are affected by the intensity of light, nitrogen concentration, and pH of the culture medium (Buxton, 1955).

2.2.3 Secondary characteristics

In the process of species identification and delimitation, secondary characteristics such as pigmentations, growth rates, and secondary metabolites are considerably important. The most widely used by researchers for secondary characteristics is pigmentations. Under fixed condition, the colors of pigmentation are taken after a week of incubation (Leslie & Summerell, 2006).

Although the colors of pigmentation are widely used, it is not a diagnostic character.

Another commonly used secondary characteristic is the growth rates. A growth rate of an isolate is measured after three days of dark incubation on PDA at either 25°C or 30°C (Burgess *et al.*, 1994). Nonetheless, Leslie & Summerell (2006) did not heavily rely on this character. Besides pigmentation and growth rates, secondary metabolite profiles are considerably useful to distinguish some species (Leslie & Summerell, 2006). However, there is still lack of information on the profiles because most of the studies done were on temperate isolates (Salleh, 1998).

2.3 Distribution and Diversity of *Fusarium* Species

Fusarium species is well distributed across many geographical regions and substrates, and also widely distributed in soils, plants, and air (Booth, 1971; Burgess *et al*., 1994; Nelson *et al*., 1994; Summerell *et al*., 2003; Salleh, 2007). Some species distributes in cosmopolitan geographic region whereas some species occur predominantly in tropical and subtropical regions, or cool to warm temperate regions (Table 2.2) (Burgess *et al*., 1994). Moreover, *Fusarium* species are even found in the enclosed buildings such as offices and hospitals (Salleh and Nurdijati, 2007). Types of vegetation are a factor for the occurrence of *Fusarium* species such as rice, beans, wheat (Lim, 1967; Hestbjerg *et al.*, 1999; Beth *et al*., 2007). Temperature in different climatic regions also affects the species distribution and virulence (Sangalang *et al*., 1995a; Saremi *et al.*, 1999). For example, when the temperature is low, the *Fusarium* disease

affecting alfalfa was increased (Richard *et al*., 1982). In Malaysia, there are at least 43 species that have been identified and isolated from various sources such as tobacco, rice, asparagus, banana, sugarcane, grass, soil, and several others (Salleh, 2007). Furthermore, five species of *Fusarium* was isolated from rice field soil in California by Lim (1967) including *F. moniliforme* (now known as *F. fujikuroi*) which is the first report of its species to be isolated from soil. However, a higher diversity of *Fusarium* species is found in rice with infection of bakanae disease in Malaysia with ten species (Nur Ain Izzati *et al*., 2005).

Table 2.2: The occurrence of some *Fusarium* species in relation to climate (Burgess *et al*., 1994)

Species which occur in most climatic regions	Species which occur mainly in temperate regions	Species which occur mainly in subtropical and tropical regions
F. chlamydosporum	*F. acmuminatum*	*F. beomiforme*
F. equiseti	*F. avenaceum*	*F. compactum*
F. proliferatum	*F. crookwellense*	*F. decemcellulare*
F. oxysporum	*F. culmorum*	*F. longipes*
F. poae	*F. graminearum*	
F. semitectum	*F. sambucinum*	
F. solani	*F. sporotrichioides*	
F. tricinctum	*F. subglutinans*	

2.4 *Fusarium* Species as Soil-borne Fungi

2.4.1 Distribution and diversity

Fusarium is known as soil-borne fungi because the genus is commonly found in soils and very widely distributed in soils across geographical region (Burgess *et al*., 1988; Burgess *et al*., 1994; Sangalang *et al*., 1995). About 14 species is recovered by Burgess *et al.* (1988) by using a debris plating technique in the soils of eastern Australia. In France, the genetic populations of *F. oxysporum* are highly diverse within soils and differentiated between soils (Edel *et al.*, 2001). Soil physical and chemical properties also affect the abundance of *Fusarium* species For instance, the levels of *F. solani* f. sp. *phaseoli* are lower when soil pH decreased and the levels of Ca, Mg, K, and P reduced (Beth *et al.*, 2007). Furthermore, the physical and chemical properties are correlated with suppression of *Fusarium* wilt of banana in Central American banana plantations (Smith and Snyder, 1971). By manipulating soil amendments, soil pH, and soil water supply, banana wilt caused by *F. oxysporum* f. sp. *cubense* can be suppressed (Peng *et al*., 1999). In addition, leguminous cover-plant, *Pueraria javanica*, increases the level of soil suppressiveness which effects the population and densities of *F. oxysporum* (Abadie *et al*., 1998). Temperature and availability of water also affect the distribution and population of *Fusarium* species in soils (Sangalang *et al*., 1995b).

2.4.2 Studies in Malaysia

Zunaidah (1984) has isolated three species of *Fusarium* from three types of vegetation; orchard, vegetable farm, and neglected soils. The pathogenicity test that was carried out showed that the isolates were saprophytes. The first intensive study on diversity of *Fusarium species* in Malaysian soil was conducted by Lim (1971). He has isolated eight species from 30 areas studied. Subsequently, the most wide spread species were *F. solani* followed by *F. oxysporum* and the rest. Furthermore, only six percent of the isolates tested were pathogenic. The latest study was done by Nik Mohd Izham *et al.* (2005) on the diversity of *Fusarium* species in the soils of Penang Island, where he obtained five species from various types of soils.

2.4.3 Life cycles in soil

Fusarium species adopted two modes of nutrition which are saprotrophs and facultative pathogens with saprotrophic phases. Plant debris in soils plays a very important role as nutrient reservoir for *Fusarium* species to continue living in soils as saprotrophs (Burgess, 1981; Burgess *et al*., 1988). A fungus needs three attributes to be consistently isolated from soils i.e. the spores must be able to commence activity, the mycelium must make successful vegetative growth, and the fungus must be able to survive in any minimal conditions (Park, 1955). There are two phases of existence in the soil for fungi i.e. an active growth phase and a survival phase (Sangalang *et al*., 1995b). An active growth phase is when the soil environment and the remained substrates are suitable with enough nutrients. On the other hand, a survival phase is when the soil conditions and environments are harsh with fewer nutrients. In the survival

phase, soil fungi such as *F. oxysporum* will form dormant structures which are chlamydospores. Other dormant form is the multicellular resting bodies known as sclerotia. During this dormant stage, *Fusarium* species implies minimal respiration rate and reserve nutrients accumulated in the mycelium that results in maximum longevity of survival (Garrett, 1981). Some *Fusarium* species produces no resting bodies and survives by continuing through slow saprophytic activity within the colonized substrate. In addition, the survival of plant pathogenic *Fusarium* in the soils continues in the residues left after harvest of a diseased crop (Garrett, 1981).

2.4.4 Isolation from soils

There are many techniques to isolate soil fungi. The soil dilution plate technique was first developed for the isolation of bacteria, but it has been successfully applied on soil fungi which give quantitative results (Warcup, 1955; Gordon, 1956; Garrett, 1981). Similarly, suspension-plating method is used for estimation of *F. oxysporum f. melonis* population in soils (Paharia & Kommedahl, 1954; Wensley & Mckeen, 1962). The screened immersion plate technique gives a wider range and variety of fungal species isolated from soils (Chesters & Thornton, 1956). On the other hand, direct soil plating method gives an advantage of detecting low fungal population in soils (Reinking & Wollenweber, 1927; Warcup, 1950). Moreover, *Fusarium* species could also be isolated by using living root or strerile straw baiting techniques e.g. peas, flax, grass, banana tissue, and wheat straw (Park, 1958; Burgess *et al*., 1994). However, plating of soil dilutions or individual soil particles spread onto nutrient agar is performed by many researchers in general (McMullen & Stack, 1983a;

Parkinson, 1994). Comparatively, debris isolation technique gives a higher diversity of *Fusarium* species recovered (McMullen & Stack, 1983a; 1983b; Burgess *et al*., 1988). The use of Modified Nash and Snyder's Medium (MNSM = PPA) is effective to determine the population of *F. solani* f. sp. *glycines* in soybean soils (Cho, 2001), while Komada's medium is selective for *F. oxysporum* (Komada, 1975). In addition, the use of PPA media is effective for isolation of *Fusarium* species (McMullen & Stack, 1983a; 1983b; Rabie *et al*., 1997).

2.4.5 Preservation

There are several techniques to preserve *Fusarium* cultures into a collection. Sterilized carnation leaf pieces are good substrates for long term preserving cultures of *Fusarium* species that was kept at -30°C (Fisher *et al*., 1982). A spore suspension in sterilized 15% glycerol kept in deep-freezer at 70°C has also been used for preservation (Leslie & Summerell, 2006). The isolates that are preserved by using this method could remain viable up to 10 years. However, lyophilization preservation technique could maintain the viable cells for an extended period of time for more than 20 years. Lyophilization preservation technique is done by freeze-drying the culture with a colonized leaf pieces (Tio *et al*., 1977). Another method used to preserve the cultures is soil preservation (Leslie & Summerell, 2006). The soil must be sterilized completely in order to preserve the *Fusarium* species This method is also considered as a long term preservation technique.

2.5 Importance of *Fusarium* species

Fungi are important organisms to be identified and studied as mentioned by Hawksworth (1991), “the world’s undescribed fungi can be viewed as a massive potential resource which awaits realization.” *Fusarium* species has caused diseases in many economically important host plants worldwide i.e. banana, cotton, legumes, maize, rice, wheat, and others (Summerell *et al*., 2003). In Malaysia, many economically important crops also have been infected by *Fusarium* species (Table 2.3). *Corynebacterium insidiosum*, the caused of bacterial wilt on alfalfa is inhibited by the presence of *Fusarium oxysporum* f. sp. *medicaginis* that is capable of producing enniatins (an antibiotic described as mycobactericide) (Johnson *et al*., 1982). Because of the serious wilt diseases caused by *F. oxysporum*, many researchers are searching for the best method to control the disease such as biological control, ecological control, and other techniques (Tamietti & Valentino, 2005). Pigeonpea wilt is caused by *Fusarium udum* in India (Prasad *et al.*, 2002). In Mexico, *Fusarium oxysporum* f. sp. *citri* seriously cause wilt and dieback of Mexican lime (*Citrus aurantifolia*) (Timmer, 1982).