MOLECULAR CHARACTERIZATION OF *Fusarium oxysporum* f. sp. *cubense* (FOC) IN MALAYSIA

LEONG SAU KUEEN

UNIVERSITI SAINS MALAYSIA

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by

LEONG SAU KUEEN

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
CLA	Carnation leaf agar
DC	Dice Coefficient
dNTPs	Dinucleotide triphosphates
ERIC	Enterobacterial Repetitive Intergenic Consensus
EtBr	Ethidium bromide
FOC	*Fusarium oxysporum* f. sp. *cubense*
f. sp.	formae specialis
het	heterokaryon incompatibility
IGS	Intergenic Spacer
ISSR	Inter-Simple Sequence Repeat
ITS	Internal Transcribed Spacer
JC	Jaccard's Coefficient
$MgCl_2$	Magnesium chloride
mtSSU	Mitochondrial small subunit
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPA	Peptone Pentachloronitrobenzene agar
PSA	Potato sucrose agar
RAMS	Random Amplified Microsatellites
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SMC	Simple Matching Coefficient
SSR	Simple Sequence Repeat
STR4	Subtropical race 4
TBE	Tris Borate EDTA
TE	Tris EDTA
TEF-1α	Transition Elongation Factor-1α
TR4	Tropical race 4
UPGMA	Unweighted Pair-group Method with Arithmetic Averages
VCG	Vegetative Compatibility Group
WA	Water agar

PENCIRIAN SECARA MOLEKUL *Fusarium oxysporum* f. sp. *cubense* (FOC) DARI MALAYSIA.

Abstrak

Sebanyak 59 pencilan *Fusarium oxysporum* f. sp. *cubense* (FOC) dianalisis menggunakan ERIC-PCR, RAMS dan RFLP-IGS. Ketiga-tiga teknik molekul ini menunjukkan variasi intraspesifik di mana pencilan-pencilan FOC menghasilkan 34 corak jalur ERIC, 33 haplotaip IGS dan 13 – 26 corak jalur mikrosatelit. Corak jalur yang dihasilkan melalui setiap teknik adalah sangat bervariasi, namun terdapat juga persamaan corak jalur ditunjukkan oleh pencilan-pencilan FOC. Analisis berkelompok UPGMA berdasarkan ERIC-PCR (15.4% - 100%) dan RAMS (11.1% - 100%) menunjukkan kesamaan genetik yang agak sama dengan data kombinasi ketiga-tiga teknik (11.0% - 100%) manakala RFLP-IGS menunjukkan kesamaan genetik yang lebih tinggi antara pencilan-pencilan FOC, iaitu 60% - 100%. Dengan menggunakan koefisien kesamaan SMC dan JC, perbezaan pengelompokkan pencilan FOC dalam dendrogram dapat diperhatikan apabila data ERIC-PCR, RAMS dan RFLP-IGS dianalisa secara berasingan dan keseluruhan. Daripada dendrogram yang diperolehi, kebanyakan pencilan FOC termasuklah empat pencilan FOC dari Indonesia berkelompok dalam kluster I dan II tanpa mengira jenis kultivar pisang dan lokasi penyampelan. Dua pencilan *F. oxysporum* dari tanah menunjukkan kesamaan genetik yang tinggi dan sentiasa dikelompok bersama dengan pencilan FOC. Bagi dua pencilan *F. solani* (angsana dan pisang awak), corak jalur yang dihasilkan melalui ketiga-tiga teknik molekul adalah berbeza daripada pencilan FOC dan sentiasa dikelompokkan berasingan daripada pencilan-pencilan FOC. Analisis penjujukan gen TEF-1α melalui kaedah parsimoni dan kaedah penjarakkan juga menunjukkan variasi intraspesifik pencilan-pencilan FOC. Keputusan yang diperoleh

menyokong hipotesis evolusi bersama antara pencilan FOC dengan perumah pisang dan generasi liar diploid di Asia serta penyebaran melalui rizom terjangkit dan tanah yang melekat padanya.

MOLECULAR CHARACTERIZATION OF *Fusarium oxysporum* f. sp. *cubense* (FOC) IN MALAYSIA.

Abstract

A total of 59 *Fusarium oxysporum* f. sp. *cubense* (FOC) isolates were analysed using ERIC-PCR, RAMS and RFLP-IGS. The three molecular techniques showed intraspecific variation among the FOC isolates, whereby 34 ERIC banding patterns, 33 IGS haplotypes and 13 – 26 microsatellite banding patterns were generated. The banding patterns generated using each technique were highly variable, but showed similarities among the FOC isolates. UPGMA cluster analyses based on ERIC-PCR (15.4% - 100%) and RAMS (11.1% - 100%) showed similarity values very close to those generated based on combined data (11.0% - 100%) of the three techniques, while RFLP-IGS showed higher similarity values of approximately 60% - 100% among the FOC isolates. Using similarity coefficient of SMC and JC, the clustering of the isolates were slightly different when data from ERIC-PCR, RAMS and RFLP-IGS were analysed individually and collectively. Based on the dendrograms, most of the FOC isolates including four FOC isolates from Indonesia were clustered in clusters I and II regardless of the banana cultivars and locations. Two *F. oxysporum* isolates from the soil showed high genetic similarity and were always clustered together with the FOC isolates. For two *F. solani* isolates (angsana and pisang awak), the banding patterns generated using the three molecular techniques were different from those of the FOC isolates and were clustered separately from the FOC isolates. Sequence analysis of the TEF-1α gene using parsimony and the distance methods also showed intraspecific variations of the FOC isolates. The results supported the hypothesis that the FOC isolates co-evolved with the edible bananas and their diploid

wild progenitors in Asia, and dissemination was probably through diseased rhizomes and attached soils.

CHAPTER 1

INTRODUCTION

Banana is the fourth most important food crop in the world after rice, wheat and maize. In many developing countries such as Rwanda, Uganda, sub-Saharan Africa and Latin America, banana is one of the major sources of nutrition and income for the people (Ploetz, 2005). Cultivated edible bananas are inter- or intraspecific combination of *Musa acuminata* Colla (AA) and *Musa balbisiana* Colla (BB) (Holiday, 1980). Genomes A and B contribute to the sweetness and starchiness of the fruit, respectively. According to the morphological characters such as pseudostem colour, shape of the petiole canal and bract features, diploid cultivars derived solely from *M. acuminata* Colla, are designated as AA while diploid hybrids with *M. balbisiana* Colla as AB (Jones, 2000). Triploids which are bigger and sturdier in size are produced through fertilization of viable diploid eggs by haploid pollen and they produce bigger fruits. This include Bluggoe (ABB), Gros Michel (AAA), 'pisang berangan' (AAA), 'pisang raja' (AAB) and 'pisang awak' (ABB) (Ploetz and Pegg, 2000).

Banana probably originated from South East Asia where varieties of wild bananas still exist in the jungles of Malaysia, Indonesia and the Philippines (Ploetz and Pegg, 2000). Wild bananas are seedy. Through selection of parthenocarpy and female sterility, the number of seeds decreased, thus increasing the edibility of the banana (Jones, 2000). Propagation via suckers enabled the movement of edible banana cultivars from Asia to other regions of the world. The worldwide distribution of banana was believed to be associated with the migration of human population and movement of suckers. It is believed that banana was brought to Africa by the Arabs

while the Portuguese brought them to the Canary Islands. Later, Spanish and Portuguese mariners introduced the food crop to the Caribbean and Latin American countries. On these foreign lands, hybridization with indigenous cultivars gave rise to more banana cultivars with different genetic make-ups.

Today, edible bananas are cultivated worldwide in 130 countries. However, 98% of the world's banana production comes from the developing countries of the Latin America-Caribbean region, Africa, Asia and the Pacific. In Malaysia, banana is the second most important fruit crop. In 2008, about 27 940 ha of lands were planted with banana and about 270 438 metric tonnes of banana were produced (http://www.doa.gov.my). The major producers of banana are Kelantan, Terengganu, Johor, Pahang, Selangor, Perak, Penang, Kedah and Perlis (Jamaluddin *et al.*, 2001). The banana planting areas are planted with popular dessert bananas such as 'pisang berangan' (AAA), Cavendish cultivars, 'pisang mas' (AA), 'pisang embun' (AAA) and 'pisang rastali' (AAB), while important cooking bananas are 'pisang tanduk' (AAB), 'pisang raja' (AAB), 'pisang nangka' (AAA), 'pisang awak' (ABB) and 'pisang abu' (ABB) (Nik Masdek, 2004).

Banana crops are vulnerable to diseases. Among the diseases, *Fusarium* wilt or Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* (FOC) is one of the most significant diseases which threaten the banana industry. The disease was first described in Australia in 1876 (Ploetz and Pegg, 2000). However, FOC was first recognized as the causal agent of Panama disease in Cuba by Erwin F. Smith in 1910, from Cuban materials and was named *Fusarium cubense* (Ploetz and Pegg, 2000). For FOC, four races have been identified worldwide. Only three races are

pathogenic to banana cultivars. Race 1 is virulent to Gros Michel (AAB), race 2 attacks Bluggoe (ABB) and race 4 which is the most virulent among these four races, attacks the Cavendish subgroup and cultivars that are susceptible to race 1 and 2. Race 3 has been reported as a pathogen of *Heliconia* spp. and only mildly affects banana (Stover, 1972).

The worldwide distribution of banana also indicates a worldwide distribution of its pathogen and pests except for the islands in the South Pacific, the Mediterranean and Somalia (Ploetz, 2000). Following the first report of the pathogen in 1876, the disease was subsequently reported in Hawaii (1904), India (1911), Jamaica (1911), Myanmar, Thailand and Malaysia (1925), Sri Lanka (1930) and had spread to almost all commercial banana growing nations by 1932 (Singh, 2000). The usage of conventional vegetative planting materials hastened the spread of the disease when infected but symptomless suckers were planted on new lands. For example, in the Philippines, FOC race 4 was introduced via infected planting materials from Taiwan (Stover, 1986). This was particularly obvious in the effort of expanding the banana export trade which was based on the Gros Michel cultivars before 1960s.

The abandonment of infested land and increasing demand worldwide forced banana growers to cultivate the crops on new lands (Stover, 1962). However, the highly productive Gros Michel succumbed to *Fusarium* wilt in 1950s. Almost 40 000 ha of plantations based on this monoclonal cultivar were wiped out and the world banana industry was almost crippled (Ploetz, 1992; Ploetz and Pegg, 2000). Although the damage was focused on the important export cultivar, other less important cultivars such as Silk (AAB) and Pome (AAB) were also badly affected.

As a result of the outbreak, various efforts had been conducted to control the disease. These include studies on the effects of soil elements on disease development (Rishbeth, 1957), the mechanism of the host in resisting the disease, quarantine of the planting materials and the most effective method is the use of resistant cultivar namely, Cavendish, AAA. The FOC threat ceased for a short period after the Gros Michel was replaced by the Cavendish cultivar until 1965 when that cultivar was also attacked by FOC.

In 1965, a suspicious new race 4 of FOC was reported to be pathogenic to the Cavendish cultivar in Taiwan, and subsequently in Australia and South Africa in the 1970s (Stover, 1986; Ploetz and Pegg, 2000). The Cavendish cultivar was presumed to be predisposed to unfavourable conditions and low temperature had been cited as the primary cause. In South East Asia, the pathogen was reported much later in the early 1990s when outbreaks occurred in Sumatra and Peninsular Malaysia (Ploetz, 2000). The first incidence caused by FOC race 4 in Malaysia was detected in 1992 at Nam Heng plantation in Johor. Although the disease was less severe compared to those in the subtropics (Stover, 1986), the pathogen destroyed Cavendish cultivar in the absence of predisposing factors.

Race 4 in the tropics was found to be genetically and pathologically different from those in the subtropics in that it affects certain clones in the tropics, for example 'Pisang Lilin' (AA) which was not affected by subtropical race 4 (Ploetz, 2006c). Thus, it was designated as tropical race 4 (TR4) and subtropical race 4 (STR4), respectively. Su *et al.* (1986) pointed out the differences of the shape and sizes of the laciniate fringes (mycelium with a comb-like look at the edge of the colony)

formed by Taiwanese race 4 and those from Australia, the Philippines and the Canary Islands. This morphological feature was used by Qi *et al.* (2008) to identify race 4 of FOC using modified Komada's medium (K2 medium). Ploetz and Pegg (2000) suspected that FOC race 4 might have evolved independently from race 1 that existed in Taiwan, Australia and Africa as Pegg *et al.* (1995) found that the FOC in Carnavon, Western Australia was more closely related to race 1 (76%) than to race 4 (29%). The occurrence of tropical race 4 in the early 1990s in Cavendish plantations in Sumatra, Halmahera and Java in Indonesia, and Peninsular Malaysia sparked the urgency to breed for new resistant banana cultivars as the Cavendish based industry is slowly losing its battle against TR4 in the tropics (Ploetz, 2000). Breeding of resistant banana cultivars intensified when tropical race 4 of FOC was subsequently recorded in Australia, Irian Jaya, India and Taiwan (Gerlach *et al.*, 2000).

Breeding for resistant banana cultivars is a daunting task as resistant cultivars with desirable features were normally sterile while fertile parents were normally highly susceptible (Ploetz, 2006a). On the other hand, FOC is highly variable. This special form of *F. oxysporum* harbours members that belong to more than 20 Vegetative Compatibility Groups (VCGs) and of different origins (O'Donnell *et al.*, 1998; Ploetz and Pegg, 2000). Other than the identified races, Ploetz (2006b) suggested the existence of other races as there were cases where FOC attacked new cultivar combinations.

Understanding the genetic diversity of FOC is essential in the selection or breeding of resistant banana cultivars and to formulate effective disease control methods. Previous studies using VCG by Correll *et al.* (1987) and Ploetz (1990)

revealed that genetically related isolates were able to form heterokaryon. These vegetatively compatible isolates were assumed to be closely related and on a number of occasions the isolates were believed to be of the same clonal lineage (Correll *et al.*, 1987). However, VCG analysis has some limitations which include difficulty in generating *nit* mutants from some isolates, weak heterokaryon formation, self-incompatibility in some isolates, cross-compatibility between isolates from different VCGs and most importantly, it does not provide information on the extent of variation or similarity within each VCG as well as members from different VCGs (Choi *et al.*, 1997). With the development of PCR, molecular methods such as random amplified polymorphic DNA (RAPD) (Bentley *et al.*, 1995; Bentley *et al.*, 1998; Kelly *et al.*, 1994), restriction fragment length polymorphism (RFLP) of Internal Transcribed Spacer (ITS) (Paavanen-Huhtala *et al.*, 1990; Bao *et al.*, 2002) and Intergenic Spacer (IGS) (Appel and Gordon, 1995) as well as sequence analysis of IGS regions, Translation Elongation Factor-1α gene (TEF-1α) (O'Donnell *et al.*, 1998; Knutsen *et al*., 2004) and mitochondrial small subunit (mtSSU) (O'Donnell *et al.*, 1998) have been used to study the genetic diversity of *Fusarium* species as well as FOC isolates.

Some of the Malaysian FOC isolates have been studied using VCG and RAPD (Bentley *et al.*, 1995; Bentley *et al.*, 1998). However, further studies need to be conducted to elucidate their genetic diversity which will benefit the effort of disease control as well as breeding for resistant or tolerant banana cultivars. Furthermore, molecular markers for rapid and efficient identification of FOC are needed to answer any confusion in morphological identification. In the present study, Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Random Amplified

Microsatellites (RAMS) analysis, Restriction Fragment Length Polymorphism of Intergenic Spacer (RFLP-IGS) and sequence analysis of the Translation Elongation Factor-1α (TEF-1α) gene were conducted to characterize the FOC isolates according to banana cultivars and to assess the genetic relationships among FOC isolates in Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of Genus *Fusarium*

The genus *Fusarium* was first introduced by Link in 1809 as *Fusarium roseum* based on the formation of canoe-shaped conidia and non-septate spores in stroma (Booth, 1971). However, the presence of stroma was no longer accepted as an essential character in identifying *Fusarium* species, following the utilization of pure culture in *Fusarium* identification (Booth, 1984). According to modern classification, *Fusarium* is classified into class Sordariomycetes, subclass Hypocreomycetidae and order Hypocreales (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser). Although efforts have been made by researchers, hoping to find the most appropriate set of criteria for the classification of *Fusarium*, the taxonomy of the genus has yet to be finalized due to the different characterization criteria adopted by different workers as well as the variations caused by cultural and environmental factors. Many of *Fusarium* species are still poorly defined.

In 1935, Wollenweber and Reinking developed a system which formed the basis for modern taxonomic system for *Fusarium*. In their monograph Die Fusarien, they classified the genus into 16 sections, 65 species, 55 varieties and 22 forms based on differences between strains rather than the similarities. The study of the isolates was done using eight different media, such as carrot agar, oat meal agar and a mixture of rice, alfalfa and barley which caused cultural variation (Nelson *et al.*, 1983). The isolates studied were classified into 16 sections based on the shape of macroconidia, the shape of the macroconidial foot cell, presence or absence of chlamydospore, the

location of the chlamydospore (intercalary or terminal), presence or absence of microconidia and the shape of microconidia.

In 1940s and 1950s, Snyder and Hansen developed another taxonomic system consisting of nine species and 34 forms (most of the species were *F. oxysporum*) by using cultures derived from single spore under controlled conditions. Their work focused on the similarities rather than differences between the isolates, which was in contrast with Wollenweber and Reinking's work. The species recognized based on Snyder and Hansen's system were *F. solani, F. oxysporum, F. roseum, F. moniliforme, F. nivale, F. tricinctum, F. rigidiuscula, F. lateritium and F. episphaeria.* Snyder and Hansen's work demonstrated that only cultures derived from single spores could be used in species identification. Their classification of *F. oxysporum* and *F. solani* were widely accepted (Nelson *et al.*, 1983).

Raillo (1950) developed 55 species classification system using new criteria namely, usage of cultural variation, presence of sclerotia and conidium ontogeny to differentiate special forms; the incurvature of macroconidia, width of conidia, number of septa and length of apical cell to differentiate sub-species and varieties; and the shape of apical cell as the major feature for speciation. In Raillo's work, single spore cultures were used to study the variation (Toussoun and Nelson, 1975).

In 1955, Bilai introduced another taxonomic system which consisted of nine sections, 26 species and 29 varieties. Bilai's work focused on the effects of environmental and cultural factors particularly to the effects of temperature, moisture, length of growth period, medium composition and germination method and

aging of conidia on the morphological appearance of *Fusarium*. In Bilai's study, it was found that different combinations of the parameters gave rise to morphological variations in *Fusarium*. However, the classification system which combined section Liseola with Elegans and Gibbosum with Discolor was only used in Russia as other taxonomists found it hard to understand.

In 1968, French scientists Messiaen and Cassini introduced another system of nine species. They followed Snyder and Hansen's system but sub-species identification of *F. roseum* was based on botanical variation rather than cultural variation as suggested by Snyder and Hansen.

Booth's work in 1971 was an important milestone in the classification of *Fusarium* species by introducing the importance of the teleomorph stage. Other important contributions include providing additional information on conidiospores as well as the emphasis on the importance of the conidiogenous cell and conidium ontogeny in *Fusarium* taxonomy. Booth also pointed out the importance of microconidial length and shape in differentiating *F. oxysporum*, *F. solani* and *F. moniliforme*. However, other researchers found the system too detailed and complex to be used (Salleh and Mushitah, 1991).

Gordon (1952) who was based in Canada also contributed to the taxonomic system of *Fusarium* in which he considered teleomorphs in the description of the fungi. Gordon studied *Fusarium* species in cereals. His approach was based on the philosophy of Wollenweber and Reinking (1935) together with some components of Snyder and Hanson's system (1940). Gordon proposed a 26 species system of the

genus *Fusarium* and modified some of the sections such as Lateritium, Martiella, Liseola and Elegans introduced by Snyder and Hansen. As a whole, Gordon's work was a compromise between Wollenweber and Reinking, and Snyder and Hansen's work (Nelson *et al.*, 1983).

Other studies following Wollenweber and Reinking's system were developed by Joffe (1974) and Gerlach (1981). Gerlach's work emphasized on the differences between the isolates cultured using eight cultural media. New species was determined based on one culture which could be a mutant. This system was again difficult to be used (Nelson *et al.*, 1983). Joffe's work introduced 13 sections, 33 species and 14 varieties of *Fusarium*. However, Nelson *et al.* (1983) pointed out that Joffe's work was a repeat of Wollenweber and Reinking's with some additional information from Gerlach's work.

The above mentioned taxonomic systems do not have an edge over the other. A combination of taxonomic systems by Nelson *et al*. (1983) introduced a system which is widely applied until today. The morphological criteria involved in this taxonomic system include the presence and absence of microconidia, the shape of microconidia, presence or absence of chlamydospore, the location of chlamydospore (intercalary or terminal), the shape of the macroconidia and the shape of the macroconidial foot cell.

2.2 *Fusarium oxysporum*

Fusarium oxysporum is an ubiquitous soil inhabiting fungus which is genetically diverse. This diverse group of fungus consists of pathogenic and non-pathogenic

strains which are morphologically indistinguishable. Pathogenicity tests need to be conducted to determine their virulence. However, researchers had speculated that non-pathogenic strains are capable of converting into pathogenic strains and vice versa (Paavanen-Huhtala *et al.*, 1990).

As the only member in the section Elegans, *F. oxysporum* grows rapidly on potato dextrose agar (PDA) with abundant aerial, white to purple mycelia. Microconidia and macroconidia are formed on short branched or unbranched monophialides. Oval to kidney-shaped microconidia of 5-16 x 2.4-3.5 µm are formed in false heads. Macroconidia of 27-55 x 3.3-5.5 µm are sickle-shaped with foot-shaped basal cells with 3-5 septa. Tan to orange sporodochia and blue sclerotia are produced. Terminal or intercalary globose chlamydospores of 7-11 µm are formed singly or in pairs in the hyphae or macroconidia. However, there are some exceptions such as *Fusarium oxysporum* f. sp. *cubense* in vegetative compatibility group (VCG) 01214 which do not produce chlamydospores (Ploetz, 2006a). Figure 2.1 shows macroconidia and microconidia produced by *F. oxysporum*.

Fusarium oxysporum exist as pathogens as well as successful saprophytes that are capable of growing and surviving on organic materials in soil for long periods. It can survive up to 30 years in the absence of a suitable host (Stover, 1962). Chlamydospores are produced as survival propagules which are heat and desiccation resistant, and could tolerate extreme climates such as in the deserts and the Antarctic (Booth, 1971). In the absence of the intended hosts, they remain dormant as chlamydospores in soil or survive as saprophytes on other plants or weeds without causing diseases.

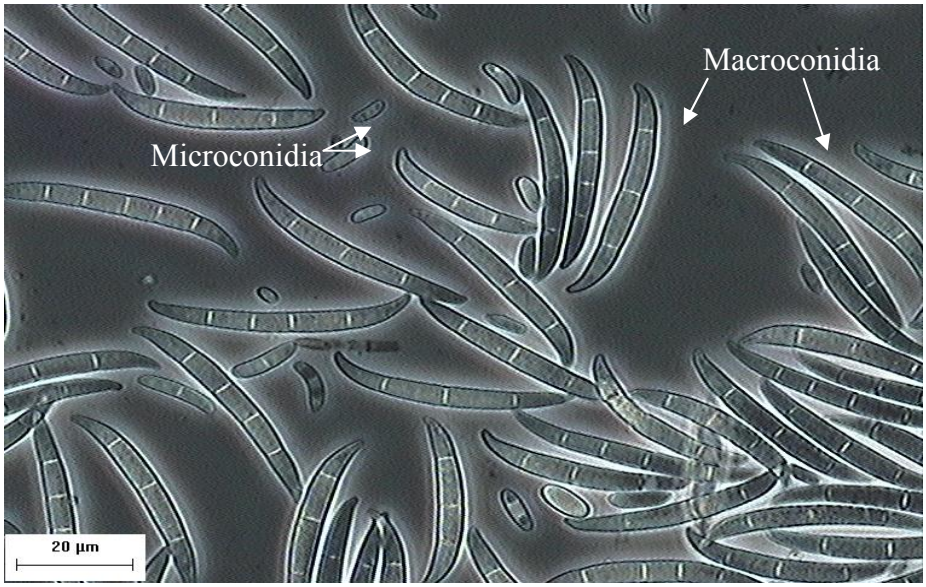

Fig. 2.1 Macroconidia with 3-5 septa and kidney-shaped microconidia.

Non-pathogenic strains have been used as biological control agents to control the pathogenic strains (Nel *et al.*, 2006a; Nel *et al.*, 2006b) and were found to be genetically more diverse than the pathogenic strains. However, studies have been focused on the pathogenic strains due to the socio-economic impact on agriculture.

Fusarium oxysporum is well known for its pathogenic strains which are responsible for vascular wilt, root rot and crown diseases in a wide range of economically important crops such as banana, tomato, asparagus and oil palm. These pathogenic strains display high level of host specificity and are categorized into more than 150 formae speciales (f. spp.) (Baayen *et al.*, 2000). Formae speciales or special forms of *Fusarium* species only infect specific host plants. For example, *F. oxysporum* f. sp. *vasinfectum*, the host is cotton; *F. oxysporum* f. sp. *melonis* only infect melons and *F. oxysporum* f. sp. *cubense* is the causal agent of vascular wilt of banana.

2.2.1 *Fusarium oxysporum* f. sp. *cubense* (FOC)

Panama disease is caused by *Fusarium oxysporum* f. sp. *cubense*, a special form of *F. oxysporum*. There are four identified races of FOC. Race 1 is virulent on Gros Michel, 'Silk' (AAB), 'Pome' (AAB), 'Pisang Awak' (ABB), 'Maqueño' (AAB) and bred tetraploid 'I.C.2' (AAAA) which was developed as a replacement for Gros Michel by the first banana-breeding programme in Trinidad. Race 2 is pathogenic on 'Bluggoe' and some bred tetraploids (AAAA) such as 'Bodles Altafort', a hybrid between Gros Michel and 'Pisang lilin' and resistant to race 1 (Ploetz, 2006b). Race 4 affects Cavendish cultivars, in addition to race 1- and race 2-susceptible clones which include genotypes AAA, AAB, AA, ABB and AAAA bred tetraploids (Stover, 1986). Genotypically different tropical and subtropical strains of race 4 have been recognized. Race 3 was reported to be virulent on *Heliconia* spp. and has mild effects on banana. However, Ploetz and Pegg (2000) suggested that other races of FOC might exist. New pathotypes might occur as a result of selection by planting resistant cultivar, either introduced or originated locally from preexisting pathogenic strain, or selection from the local population of non-pathogenic strains. Relatively simple genetic exchange in one pathogenic race might give rise to a new race (Gordon and Martyn, 1997).

Fusarium oxysporum f. sp. *cubense* is regarded as a highly complex pathogen. One of the methods used to estimate genetic diversity of FOC was using Vegetative Compatibility Group (VCG). Vegetative Compatibility Group is based on the abilities of the isolates to form heterokaryons when paired. Vegetative compatibility in *Fusarium* species is determined by a set of vegetative incompatibility (*vic*) or heterokaryon incompatibility (*het*) loci. Heterokaryon will only be formed when two

interacting strains carry the same alleles at all *vic* loci (Puhalla, 1985). To date, for FOC there are at least 24 VCGs identified (Ploetz and Corell, 1988; Bentley *et al.*, 1998) in which 11 of them are found in Malaysia (Bentley *et al.*, 1999). Table 2.1 shows VCGs of FOC in Asia.

Table 2.1 Vegetative Compatibility Groups of FOC strains in Asia (Bentley *et al.*, 1999).

Country	VCG	Number of VCG
Australia	0120, 0124, 0124/5, 0125, 0128, 0129, 01211, 01213/16, 01220.	8
Indonesia	0120, 0120/15, 0121, 0124/5, 0126, 01213, 01213/16, 01215, 01216, 01218, 01219.	11
Malaysia	0120, 0121, 0123, 0124/5, 0125, 01213, 01213/16, 01216, 01217, 01218, 01222.	11
Philippines	0122, 0123, 0126.	3
Taiwan	0120, 0121, 0123, 01213.	4
Thailand	0123, 0124, 0124/5, 01218, 01220, 01221.	6

2.2.2 Symptoms of Panama Disease

Disease infection can be divided into three fundamental steps namely, colonization, invasion and multiplication of hyphae and spores. Upon stimulation by host roots exudates, the germinated hyphal element penetrates lateral roots and colonizes the root tissues. At this stage, wounded roots are easily penetrated by the fungus. Often, wounded roots caused by nematodes (*Radopholus similis*) may contribute to the infection of FOC on banana roots (Holiday, 1980).

According to Booth (1971), banana plants of all ages were susceptible. Symptoms were normally visible on 5 months old plants although 2–3 months old plants were also killed under favourable conditions (Singh, 2000). According to

Stover (1972), suckers aged less than 4 months old and 5 feet tall do not show external symptoms.

The infected plants show internal and external symptoms as a result of the hosts' reactions to invasion. The first internal symptom of *Fusarium* wilt is vascular discolouration which begins with the yellowing of a few vascular strands in the rhizome and is most prominent where the stele joins the cortex (Ploetz, 2006a). As the disease advances, the vascular tissues change into reddish to dark brown discolouration (Ploetz, 1992) (Fig. 2.2). In some cases, as a result of host defence mechanism, formation of gel or tyloses in the vascular system causes necrosis. However, internal symptoms may vary, depending on the fungal strains and the presence of bacteria or nematodes in the vascular element (Booth, 1971).

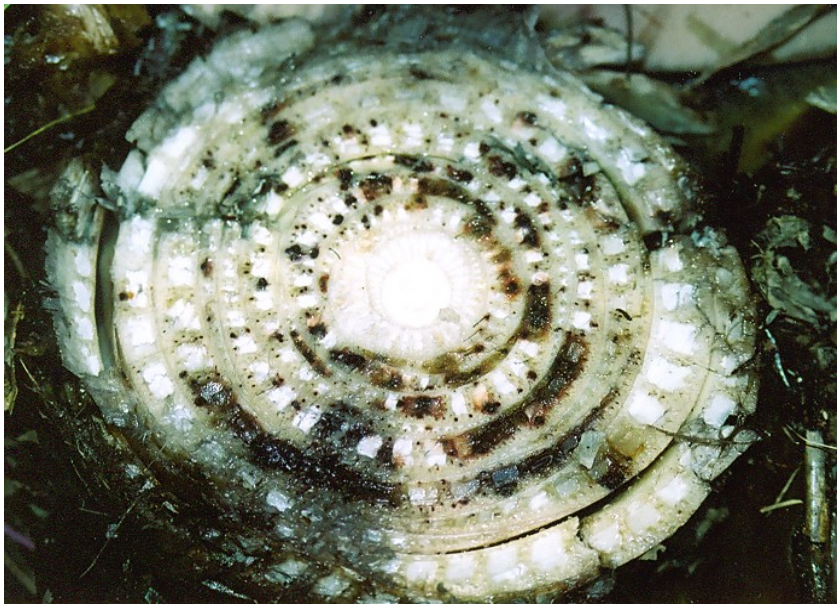

Figure 2.2: The vascular system (xylem) showing discolouration and was brown in colour when the pseudostem was cut horizontally.

The first external symptom is yellowing of the oldest leaves due to nitrogen deficiency (Schumann, 1998) and the longitudinal splitting of the lower portion of the outer leaf sheaths on the pseudostem 2 months after initial infection (Holiday, 1980), followed by wilting and collapse of the leaves at the petiole base (Stover, 1972). Growth does not cease immediately, new leaves produced are blotchy yellowish, and laminas are usually distorted and wrinkled (Simmonds, 1966). At a later stage, younger leaves collapsed and pronounced vascular discolouration is evident when pseudostem was cut horizontally. Finally, the plant dies due to wilting of the leaves (Fig. 2.3) and rotting of the roots. The plant remains standing for 1-2 months before decaying and eventually it topples (Ploetz, 1992; Singh, 2000).

Figure 2.3: Diseased banana showing wilting and yellowing of leaf sheaths and the outer leaf sheaths died off.

Despite the external and internal symptoms of the plant, no wilting or discolouration is observed on the fruits. This differed from Moko disease caused by race 2 of *Ralstonia solanacearum* which results in wilting and chlorosis in suckers aged less than 4 months old and internal discolouration of the berries (Ploetz, 2005). Although the affected suckers may still produce fruits, the bunches develop abnormally and ripen prematurely or irregularly (Ploetz, 1992; Singh, 2000). As for plants infected by FOC, the fruits produced were affected in terms of appearance, shelf life and marketability. Moreover, when the plants were killed, the yield decreased (Ploetz, 2005). Furthermore, the infested lands were hard to be reclaimed and virgin lands were less available, causing further decrease in banana production.

2.2.3 Disease Cycle and Development

In infested cultivated lands, FOC can be recovered in abundance in the form of chlamydospores. The resting bodies were heat resistant and can survive in soil in the absence of the intended hosts for up to 30 years (Ploetz, 1992). In addition, the ability of chlamydospores to perpetuate on roots of certain weeds such as *Cyperus iria* L., *C. rotundus* L., and *Fimbristylis koidzumiana* Ohwi without causing disease also ensure the survival of FOC in soil in the absence of suitable hosts (Su *et al.*, 1986).

Chlamydospore germination was stimulated by root exudates and the process was carbohydrate dependent (Cook and Schroth, 1965; Griffin, 1969). This was particularly important for complete germination at high spore densities in axenic culture and soil (Cook and Schroth, 1965; Griffin, 1969; Griffin, 1970). Upon germination, the germ tube adheres and penetrates through the lateral roots. Direct

penetration through pseudostem does not occur. In the presence of burrowing nematodes (*Radopholus similis*), wounded roots were readily penetrated (Holiday, 1980). Although mechanical wounds increased the infection rate or shorten the incubation period but they were not vital in successful penetration (Singh, 2000).

In vascular tissues, FOC mycelia proliferate and produce microconidia which were transported upwards to facilitate the spread of the fungus within the vascular vessel. At the end of the vessel, the movement of microconidia was blocked by perforation plates. Gel was formed at the end walls as part of host defence mechanisms. In resistant hosts, the gel formed will persist long enough until the xylem was completely occluded by tyloses. In susceptible cultivars, the gel degraded before tyloses can be formed and allowed the trapped microconidia to germinate. Systemic infection occurs when the newly germinated mycelium penetrates through pit openings and new spores were formed and translocated to the upper plant parts. The pathogens were not capable of invading living tissues; they grow in the water-conducting vessels. Only at advanced stages of disease development, the mycelia penetrate into adjacent parenchyma tissues and chlamydospores were formed while formation of macroconidia can be seen on leaves and petioles (Stover, 1972; Deacon, 1997).

Chlamydospore formation was found in abundance in the senescent epidermis-cortex tissues, but not in the vascular system of the host plant (Trujillo and Snyder, 1963; Ploetz, 2000). When the host plant dies, the resting bodies returned to the soil as a result of decomposition. In the absence of a suitable host, the chlamydospore would continue to survive and new chlamydospores were formed via the germination

of the old ones and from macroconidia (Trujillo and Snyder, 1963) when the conidial density in soil was high (Schneider and Seaman, 1974). Due to the accumulative effects of self inhibitor, germination of macroconidia were suppressed (Robinson and Park, 1966; Griffin, 1969; Robinson and Garrett, 1969; Griffin, 1970) and for continuous survival, macroconidia were converted into chlamydospores. Therefore, it is hard to reclaim the infested lands. This decreases the land available for banana planting which eventually result in the decreased production of banana. The cycle repeats when the chlamydospores were stimulated to germinate by root exudates.

2.2.4 Defence Mechanisms in Banana

Every plant has its innate chemical and cellular barriers which protects the host from the invasion of pathogens. Generally, the defence mechanisms are triggered off by recognition of non-self substance invading the host. These non-self substances could be toxins produced by the pathogens which lyse the outer layer of the host in facilitating the invasion of the mycelium or the lysed fragments of the pathogen itself. Once the cellular defence system inside the banana plant recognized the pathogen, gel formation followed by tyloses occlusion will block further advancement of the mycelium and spores which also kill part of the host plant (Kendrick, 1992; Deacon, 1997).

Successful penetration of FOC does not necessarily result in disease formation. Inside the hosts, there were a series of defence mechanisms which protects the hosts from diseases. In the vascular system, the first physical barrier is the end walls of the perforation plates. Microconidia in the transpirational stream were trapped by the perforation plates end walls. After that, gel primarily composed of pectins, calcium

pectates, hemicellulose and traces of protein were formed as a result of the swelling of the perforation plates, the vessels end walls and side walls (Beckman and Zaroogian, 1967).

In resistant cultivars, further movement of the pathogen was prevented by the formation of gel within 24 – 48 hours, followed by tyloses formation after 48 – 96 hours before the gel degrades (Ploetz, 2000). According to Schumann (1998), tyloses were formed as a result of poisoning of parenchyma cells which balloons into the vessels through cell wall pits. These rapid responses of the hosts prevent further colonization of the pathogen by localizing the pathogen. In susceptible hosts, the gel degrades before the vessel can be seal-off by tyloses (Stover, 1972). As a result, the spores were transported to the upper part of the hosts via translocation and the infection became systemic.

2.2.5 Disease Control

Since the first epidemic in Panama, numerous control measures have been tested in the effort to control the disease. These efforts include biological and cultural control which was designed to reduce the susceptibility of the host to infection and to reduce the pathogen population if not eradicating them. Cultural control include the eradication of diseased plants and their immediate neighbours, quarantine and utilization of pathogen-free planting materials, soil amendment and usage of disease suppressive soil, chemical fumigation as well as flood fallowing and crop rotation. However, these measures merely delayed the disease and gave temporary relieve to the industry (Simmonds, 1966). The effort which harbours the greatest hope of

combating the disease is breeding of disease resistant banana cultivars (Stover, 1986).

At the early stage of Panama disease outbreak, quarantine and eradication of infected planting materials were some of the early efforts to control the disease (Stover, 1972). Planting materials were quarantined and carefully inspected before being planted in the field and diseased banana plant and its immediate neighbours were cut down and buried or discarded in a safe manner. In Jamaica, the diseased plant and the neighbouring banana plants were cut down and drenched in heavy soil. However, this method only managed to slow down the spread of the disease as immediate control was not taken and the spread of pathogen went beyond the treatment area before apparently healthy plants were destroyed (Simmonds, 1966).

Usage of disease suppressive soils was successful in some areas. However, it depends on the length of the period in which high production could be maintained in the presence of the pathogen. Furthermore, disease suppressiveness could not be transferred to disease condusive soils (Ploetz, 2006a). In Central America, chemical analyses of the soils revealed that disease suppressive soils were high in montmorillonite clays, in contrast to the disease condusive soils which contain higher proportion of kaolinite clay. Presence of antagonistic microorganisms contributes to the effectiveness of suppressive soils. The montmorillonite clays hold higher nutrient content and created more favourable pH for antagonistic microorganisms (Deacon, 2006). For example, the non-pathogenic strains of *F. oxysporum* were found to be competing for organic substrates with the pathogenic strains in disease-free vegetable farms in Chateaurenard, France. On the other hand, higher inoculum level is also

required to cause disease in disease suppressive soils as chlamydospores germinate poorly and the population of the pathogen declines more rapidly (Deacon, 2006). However, disease development was also influenced by other environmental factors (Ploetz, 2000). These factors include soil pH, drainage of the soil, health condition of the hosts, types of banana cultivar planted and also the weather. Acidic soils (pH 6 and below) were more favourable for disease development. According to Simmonds (1966), long live banana cultivation soils in Jamaica and Central America usually showed neutral or slightly alkaline pH. Stover and Malo (1972) examined the factors associated with the occurrence of *Fusarium* wilt in resistant Dwarf Cavendish in Gran Canaria and found that breakdown of resistance were due to the adverse growing conditions which include impermeable soil, poor drainage, low seasonal temperature and acidic soil pH. They concluded that these factors, in combination, impeded the optimum development of the banana which in turn affects the full expression of disease resistance in the otherwise resistant Dwarf Cavendish. Deacon (1997) also pointed out that *Fusarium* wilt is more severe under suboptimal conditions as host resistance might be suppressed and therefore, infection could occur in resistant cultivar in such conditions.

Chemical fumigation and utilization of fungicide has not been successful in the attempt to control the disease. Methyl bromide was unsuccessful due to the resurgence of the FOC after a short period. In Africa, the disease made a come back after 3 years of using methyl bromide. Plant injection using Carbendazim and potassium phosphonate were unrepeatable although these chemicals provide certain degree of control (Ploetz, 2000).

Flood fallowing has been proven to be detrimental for the survival of FOC by depriving its oxygen supply. Low partial oxygen pressure and effects of toxins such as acetic acid were presumed to be the factors which eliminated the pathogen. Furthermore, continued germination of conidia in submerged soil was promoted by carbon dioxide (CO_2) and thus suppressing the formation of chlamydospores when the germinated colonies were subsequently killed by the anaerobic condition (Meredith, 1970). Flood fallowing was first carried out in Honduras in 1939. It was designed to flood the infected land for a period sufficiently to reduce the soil population of the pathogen, if unable to destroy them (Simmonds, 1966). It has provided temporary success in some areas. In Taiwan, crop rotation with paddy for 1 and 3 years successfully reduced the disease incidence from 40% to 12.7% and 3.6%, respectively. It has been used as a common practice to reclaim infested fields in Taiwan for banana production (Su *et al.*, 1986). However, this control measure was not without flaw. Flood fallowing was only applicable to flat lands, and it is also expensive and demanding as very large and regular water was needed to compensate for the water loss through seepage and evaporation (Simmonds, 1966).

Based on the fact that Panama disease was disseminated through diseased rhizomes, pathogen free planting materials are important in establishing new plantations, especially when disease free rhizomes were hard to obtain from the field. Therefore, tissue cultured plantlets can be used in new planting lands. These plantlets were well established in the field and mature plants with uniform growth produced normal fruit with shorter harvest period (Su *et al.*, 1986). However, preparations of tissue cultured plantlets are expensive and small-holder farmers could not afford it. It has been suggested that tissue cultured plantlet can be used to produce disease free