MOLECULAR ANALYSIS OF *Fusarium oxysporum* f. sp. *cubense* ISOLATES AND DEFENSE GENE EXPRESSION IN BANANA INFECTED WITH *Fusarium* WILT

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MOLECULAR ANALYSIS OF *Fusarium oxysporum* f. sp. *cubense* ISOLATES AND DEFENSE GENE EPRESSION IN BANANA INFECTED WITH *Fusarium* WILT.

ABSTRACT

The Panama disease of banana, that blocks the xylem from transporting water and nutrients, is transmitted by the soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense (Foc)*. Knowledge of the characteristic and morphological behavior of the fungal population is crucial to develop strategies to reduce the damage by the disease. At present, there is no effective and economically safe methods are available to protect crops from Fusarium wilt disease. The identification of *Fusarium* species is also challenging as it relies on the slight differences in its morphology. Different cultural conditions can cause similar species to diverge. Additionally little is known about the genetic and molecular basis of the pathogenic mechanism of interaction between *Foc* and banana. A reliable greenhouse bioassay is therefore needed to help gain deeper insight on *Foc*-banana interactions. A standardized bioassay is an essential pre-requisite for biological control studies and epidemiology research. On top of that, progressive increase of invasive outbreaks and reports of *Fusarium* species further emphasize the necessity for a rapid, practical and reproducible bioassay protocol. Inoculum standardization is an important parameter in such procedures as optimal desired concentration and reproducibility of spore suspension may influence the bioassay results. This study focused firstly on the morphology, physiology and the pathogenic effects of *Foc* available in our collection. We aimed to study the variability of the pathogen's phenotypic characteristic with its pathogenicity towards the host. Additionally we also developed a fast and precise method of evaluating the cell-density of *Fusarium* spore suspension in a cv. 'Berangan' bioassay experiment. We then described a simple standardized workflow and procedures for

testing Fusarium wilt disease response in *Musa acuminata* using cv. 'Berangan' of tissue-culture origin as a model. In our experiment, we found that desired spore concentration of 1 x 10^4 to 10^6 were obtained after 7 day-incubation in the dark at 26°C to 28°C with two times of shaking per day. These optimised conditions are proposed as guidelines for a reference inoculum preparation method in subsequent bioassay experiments. Interestingly, during infection of Berangan with *Foc* using root-immersed approaches, phenotypic assays were able to detect physical symptoms as early as 2 days post-inoculation and molecular detection was able to detect differences in gene expression as early as 2 hours post infection in a standardized challenge assay. Berangan was found to be highly susceptible to *Foc*R4 (C1 HIR isolate) with LSI and RDI scores of 3.74 and 4.39 respectively. The molecular approach detected differential expression of *pathogen related protein 10 (PR10)* and *phenylalanine ammonia lyase (PAL)* genes that showed a measureable transcript of RNA at day 0 followed by signature changes as early as day 2 and day 4. We also describe a procedure for extracting good quality RNA from healthy and infected banana plantlets for RT-qPCR analysis. *RPS2* was validated as the most stable reference genes for normalization of defense-related genes in RT-qPCR. The analyses results showed that, the expressions of *PR10* and *PAL* genes were upregulated upon infection by *Foc*, which indicated the activation of defense responses in the banana-*Foc* interaction.

Keywords: Panama disease, Host-pathogen interaction, Bioassay experiment, Reverse-Transcriptase quantitative PCR, Disease resistance gene

PENCIRIAN DAN ANALISIS MOLEKUL PENCILAN *Fusarium oxysporum* f. sp. *cubense* DAN EKSPRESI GEN PERTAHANAN DALAM PISANG YANG DIJANGKITI PENYAKIT LAYU *Fusarium*

ABSTRAK

Penyakit Panama adalah disebabkan kerana penyekatan yang berlaku di xylem dari menyalurkan air dan zat makanan, yang di sebabkan oleh kulat bawaan tanah, *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Pengetahuan tentang ciri-ciri dan morfologi patogen adalah penting untuk mengurangkan kerosakan oleh penyakit bawaannya. Sehingga kini, tiada langkah dan cara yg selamat untuk mengelakkan tanaman daripada penyakit layu Fusarium. Proses mengenalpasti spesies *Fusarium* sangatlah mencabar kerana ianya bergantung kepada perubahan kecil pada morfologinya. Keadaan kultur yang berlainan boleh menyebabkan spesis yang sama untuk mencapah. Tambahan pula, tidak banyak yang diketahui tentang genetik dan mekanisma molekul pada *Foc* yang menyebabkan penyakit kepada pokok pisang. Oleh itu, penilaian-biologi rumah hijau dipercayai diperlukan untuk membantu mendapatkan maklumat yang lebih mendalam mengenai interaksi antara *Fusarium* dan pisang. Penilaian-biologi yang seragam adalah pra-syarat penting bagi kajian biologi kawalan dan penyelidikan epidemiologi. Selain itu, penyebaran wabak dan laporan spesies *Fusarium* perlu menekankan keperluan yang cepat, praktikal dan protokol yang memberi keputusan yang tepat. Disamping itu penyelarasan inokulum adalah satu factor penting dalam prosedur seperti kepekatan optima yang dikehendaki dan pengulangan eksperimen yang konsisten bagi menghasilkan pengampaian spora yang stabil boleh mempengaruhi keputusan penilaian-biologi. Oleh itu, kajian ini menumpukan kepada kajian morfologi, fisiologi, dan kesan patogenik oleh *Foc* yang terdapat di dalam koleksi kami. Matlamat kami adalah untuk mengkaji variasi sifat fenotip patogen terhadap kesan patogeniknya kepada perumahnya. Selain itu, kami juga membangunkan satu kaedah yang cepat dan tepat untuk menilai

ketumpatan sel *Fusarium* dalam pengampaian spora kepada kaltivar 'Berangan' dalam eksperimen penilaian-biologi. Kami menyediakan aliran kerja seragam yang mudah dan prosedur untuk menguji tindak balas penyakit *Fusarium* kepada *Musa acuminata* menggunakan kultivar 'Berangan' dari tisu-kultur sebagai model rujukan. Dalam eksperimen ini, kami mendapati bahawa kepekatan spora daripada 1 x 10^4 sehingga 10^6 diperolehi selepas 7 hari pengeraman dalam gelap pada 26°C hingga 28°C dengan dua kali pergoncangan setiap hari. Syarat-syarat ujian yang dicadangkan adalah sebagai garis panduan bagi kaedah penyediaan rujukan inokulum dalam eksperimen penilaian-biologi yang berikutnya. Menariknya, hasil penyelidikan ini juga berjaya menunjukkan bahawa, semasa jangkitan Berangan dengan *Foc* menggunakan kaedah rendaman akar, ujian fenotip dapat mengesan gejala fizikal seawal 2 hari selepas inokulasi dan pengesanan molekul dapat mengesan perbezaan ekspresi gen seawal 2 jam selepas jangkitan dalam ujian yang seragam. Berangan didapati sangat mudah dijangkiti oleh *Foc*R4 (pencilan C1 HIR) dengan skor LSI dan RDI sebanyak 3.74 dan 4.39 masing-masing. Pendekatan molekul juga dapat mengesan pembezaan tindakbalas oleh '*pathogen related protein 10'* (*PR10*) dan '*phenylalanine ammonia'* (*PAL*) gen yang menunjukkan bahawa ekspresi *RNA* dapat diukur seawal hari 0 diikuti dengan perubahan tanda selepas hari ke 2 dan hari ke 4. Penyelidikan ini juga berjaya menghasilkan prosedur mengekstrak RNA yang berkualiti dari anak pokok yang sihat dan anak pokok yang telah dijangkiti untuk analisis RT-qPCR. Dalam analisis ini, keputusan menunjukkan bahawa ekspresi gen *PR10* dan *PAL* menunjukkan penambahan regulasi ketika dalam keadaan jangkitan oleh patogen *Foc* yang mana ini menunjukkan pengaktifan tindak balas pertahanan dalam interaksi pisang-*Foc*.

Kata kunci: Penyakit Panama, Interaksi perumah-patogen, Penilaian biologi, *Reverse-Transcriptase* Kualitatif PCR, Gen rintangan penyakit

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"Every accomplishment starts with the decision to try,
Thereafter success depends on combination of
insistence from the self and support from the other"

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

℃	: degree Celsius
%	: percent
µg	: microgram
µL	: microliter
µM	: micromolar
18S	: 18 sub unit
26S	: 26 sub unit
ALM	: Armstrong's liquid media
Avr	: avirulence
bp	: base pair
cDNA	: complimentary deoxyribonucleic acid
CLA	: carnation leaf agar
cm	: centimeter
Ct	: threshold cycle
cv	: cultivar
CWA	: cell wall apposition
d	: day
dpi	: day of post inoculation
DSI	: disease severity index
E	: efficiency
EDTA	: Ethylenediaminetetraacetic acid
F	: Forward
F.	: *Fusarium*
f. sp.	: formae specialis
FAOSTAT	: Food and Agriculture Organization Statistic
FESEM	: field emission scanning microscopy
Fo	: *Fusarium oxysporum*
Foc	: *Fusarium oxysporum* f. sp. *cubense*
*Foc*R1	: *Fusarium oxysporum* f. sp. *cubense* Race 1
*Foc*R4	: *Fusarium oxysporum* f. sp. *cubense* Race 4

*Foc*TR4 : *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4

Fod : *Fusarium oxysporum* f. sp. *dianthi*

Fol : *Fusarium oxysporum* f. sp. *lycopersi*

Fov : *Fusarium oxysporum* f. sp. *vasinfectum*

g : gram

GAPDH : glyceraldehyde-3-phosphate

GFP : green fluorescent protein

h : hour

HCl : hydrochloric acid

IGS : intergenic spacer

IPGRI : International Plant Genetic Resources Institute

ISR : induced systemic resistance

ITS : internal transcribed spacer

JA : jasmonic acid

K : kilo

kb : kilo base pair

LSI : leaf severity index

M : Molar

MAMPs : microbe associate molecular pattern

mg : milligram

ml : milliliter

mM : millimeter

MM : minimal media

mm^3 : cubic millimeter

NA : not available

NaCl : sodium chloride

NCBI : National Center for Biotechnology Information

ng : nano gram

nit : nitrate

nm : nanometer

OD : optical density

PAL : phenylalanine ammonia-lyase

PAMPs : pathogen-associated molecular patterns

PCR : polymerase chain reaction

PDA : potato dextrose agar

PDB : potato dextrose broth

pg : picogram

ppm : parts per million

PR10 : pathogenesis-related 10

PRR : pattern-recognition receptors

psi : pounds per square inch

PTI : PAMP-triggered immunity

R : reverse

RAPD : Random Amplified Polymorphic DNA

rcf : relative centrifugal force

RDI : rhizome discoloration index

rDNA : ribosomal deoxyribonucleic acid

RFLP : restriction fragment length polymorphism

RNA : ribonucleic acid

RNase : ribonuclease

ROS : reactive oxygen species

rpm : rotation per minute

RPS2 : ribosomal protein S2

RT-qPCR : reverse-transcriptase quantative polymerase chain reaction

SA : salicylic acid

SAR : systemic acquired resistance

SDS : sodium dodecyl sulphate

SIX : secreted-in-xylem

SIX1 : secreted-in-xylem 1

Six1a : *secreted-in-xylem 1a*

Six1c : *secreted-in-xylem 1c*

SIX2 : *secreted-in-xylem 2*

SIX3 : *secreted-in-xylem 3*

SIX4 : *secreted-in-xylem 4*

SIX6	: *secreted-in-xylem 6*
SIX8	: *secreted-in-xylem 8*
spp.	: species
SSI	: single spore isolation
SSR	: simple sequence repeats
ST4	: subtropical 4
TBE	: tris-borate-EDTA
TR4	: tropical race 4
VAGs	: virulence associated genes
VCG	: vegetative compatibility group
vic	: vegetative incompatibility loci

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Banana (*Musa spp.*) is amongst the world's most important economic and agricultural crop. It is ranked as the fourth most important staple food crop following rice, wheat and maize. Notably, banana is also a fruit in high demand which contributes to major profit for most hypermarkets, making them a significant for economic and global food security (Alemu, 2017).

One of the major global constraint in the production of bananas is the disease known as Fusarium wilt also known as Panama disease. This disease was first discovered in 1876 in Australia (Ploetz, 2015). It was later in 1910 that the soil-inhabiting fungus *Fusarium oxysporum* f.sp *cubense* (*Foc*) was recognized as the causal agent in Cuba, from which the name of the *forma speciales* was derived (Ploetz, 2005). Fusarium wilt, results in severe losses in many banana-growing countries of the world (Ploetz, 2015).

The disease starts with the fungus infecting the roots of the banana plant, colonizing the vascular system of the rhizome followed by the pseudostem area, where it actively blocks the xylem vessels, inducing a classic wilt disease, which commonly occurs after 5 to 6 months of planting. The symptoms of the disease are expressed both externally and internally (Thangavelu & Mustaffa, 2012). Generally, there are a few factors that could influence the development of the disease, such as the type of the cultivar, soil, drainage, and environmental conditions (Pérez-vicente et al., 2014).

The management of Fusarium wilt is generally through on-farm practices that include preventing the entry of the pathogen into new plantations, proper destruction of infected plants, and the isolation of susceptible plants from infested fields, are critical to minimize crop loss and prevent further spread of the pathogen (Simone & Cashion,

1996). However, there is no means of curing the Fusarium wilt disease once the plant is attacked as the pathogen's presence is in the soil itself. The pathogen could be called a "silent killer" by the way the pathogenic strains can lay dormant in the form of asexual spores (chlamydospores) which persists in the soil for up to 30 years before regaining virulence (Buddenhagen, 2009). Therefore, Panama disease was and still is a critical problem in the banana industry. It has become a very difficult problem to deal with. Chemical and fungicide have been used with little success to control this disease.

Since the Fusarium wilt is significantly influenced by the host genotype, the best means of controlling this disease is by implementing disease resistant planting materials. However to date, no established edible resistant banana cultivars are available, thus the use of genetic engineering is an important option for the generation of resistant cultivars (Ghag et al., 2014). Resistance breeding can be difficult when no dominant gene is identified. According to Diener et al. (2005), breeding for resistant plants is the most efficient measure to manage Fusarium wilt in banana plants. Agricultural research organizations worldwide are now making drastic efforts to identify and characterize resistant genes in bananas against the Fusarium wilt disease (Peraza-Echeverria et al., 2008). However, transferring disease resistance alleles to the genetic background of elite genotypes of banana through breeding programme is a complex process and time-consuming (Ortiz, 2006). One of crucial steps of the breeding programme is the selection steps to screen for Fusarium wilt resistance (Ribeiro et al., 2011).

Rapid and reliable greenhouse bioassays are required to improve the understanding of the plant's responses to *Foc* and its resistance mechanisms as well as the characterization and study of the pathogenicity mechanism in fungal populations. Banana breeding programmed carried out in 'Silk' (AAB) plants in Embrapa Cassava and Tropical Fruits to screen Fusarium wilt resistance cultivar, shows that more than

90% of plants displays high and consistent level of infection, and 10% of plants remain symptomless, suggesting the possibility of escape (Ribeiro et al., 2011). Such *Foc* bioassay must eliminate the chance of infection escape and ensure precise expression of the host plant resistance or the fungal pathogenicity itself. It must give consistent and reproducible wilting reactions of the disease, which corresponds with the known resistance among other banana cultivars. It should also be highly interrelated with field observations under natural infection environments. Currently, the selection of *Foc* resistance through field based studies can take up to several years (Krishna et al., 2016). This technique rendered variable results due to heterogeneous distributions of inoculum and interaction with other microorganism present inside the soil (Dita et al., 2010). One other bottleneck reason of banana breeding programmed is lack of knowledge regarding genetic variation through morphological characterization and molecular basis of resistance. Such studies are important and it is dependent on a reliable greenhouse bioassay for *Foc*-banana interaction (Ribeiro et al., 2011).

Information of the characteristic and morphological behavior of the fungal population is also crucial in order to minimize the damage done by the disease. Characterization of the *Fusarium* species is often quite challenging as it relies on slight differences in its morphology, as well as the different cultural conditions which can cause similar species to diverge (Doohan et al., 1998). In addition, the race concepts further complicates the identification and characterization of the fungus, which does not provide adequate capture of its genetic variations. To overcome this issue, further characterization has been implemented using vegetative compatibility group (VCG) analysis (Ploetz & Correll, 1988), coupled together with cultural and morphological characteristics (Thaware et al., 2017). At least 21 different VCGs of *Foc* have been identified and characterized, with majority of the group present in Asia (Fourie et al., 2009; Pegg & Ploetz, 1997). TR4 isolates are designated as VCG 01213 (or VCG

01216, which is a different designation for the same VCG), while isolates classified as ST4 belong to VCGs 0120, 0121, 0122, 0129 and 01211 (Buddenhagen, 2009).

Furthermore, to support morphological characterization, molecular tools have been developed. DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity of *Fusarium* species. Since the invent of Polymerase Chain Reaction (PCR) technique, which was developed in the early 1980s by Mullis, molecular biology approaches for molecular diagnostic has been revolutionized (Wong & Landsverk, 2013). Molecular markers also provide a powerful tool for population genetics studies in fungi and have been used to characterize the genetic diversity of worldwide populations of *Foc* (Visser et al., 2010). For that reason, DNA-based techniques including simple PCR assay, species specific PCR and also quantitative Real-Time PCR would be suitable to analyze and detect genetic variation within the *Foc* population and the mechanism of the pathogenicity towards banana plants.

1.2 Problem Statement and Justification

The spread of the fungal pathogen *Foc* has contributed to serious declines in the productivity of banana and plantain based farms. In Malaysia, the soil-borne pathogen is difficult to control because of its hidden status and resistance towards fungicide. Isolation and identification of pathogens is a precondition to control pathogenic diseases. Studying these pathogens and controlling them will help to increase the yield and quality of banana production, thereby supporting the supply and quenching its demand. Knowledge about the pathogens provides insights to enable the introduction of new or novel management strategies.

The present research project has been undertaken to identify and study the cultural, morphological and molecular characterization of the causative agents of vascular wilt disease of bananas (Fusarium wilt disease). Furthermore, there is no standardized challenge procedure or bioassay for assessing disease response to the *Foc* fungus. In previous studies, different inoculum concentrations, plant age, inoculation methods and disease scoring index were used for the experiments. Thus this study also presents a simple standardized workflow and procedure for testing Fusarium wilt disease response in *Musa acuminata* using *M. acuminata* cv. 'Berangan' of tissue-culture origin as a model.

1.3 Objectives

The objectives of this study are:

1. To study the morphology, physiology and the pathogenicitic effects of the Malaysian *Foc* isolates on *Musa acuminata* cv. Berangan.
2. To isolate and identify pathogenic *F. oxysporum* strain from the pseudostem and rhizosphere of banana plants from hotspot in Jeli, Kelantan.
3. To develop a fast and precise method for preparing the cell-density of *Fusarium* spore suspension for the cv. 'Berangan' bioassay experiment.
4. To design and improve bioassay protocols for early screening of Fusarium wilt disease and analysis of gene expression profile in banana roots in response to infection by race 1 and race 4 of *Foc*.

CHAPTER 2: LITERATURE REVIEW

2.1 Banana cultivation and its origin

Musa spp., both banana and plantain, represent fourth most important staple food commodity worldwide (Azad et al., 2016). Global export of banana worldwide reach the highest record of 19.2 million tons in 2018 (Food and Agriculture Organization of the United Nations, 2018). In tropical areas, it is one of the most important staple food for more than 400 million people (IPGRI, 2000). Local market banana trade is one of the few activities that provide households regular income throughout the year (Arias et al., 2003). Earliest domestication of banana had occur in South East Asia and Indochina. This is because greatest diversity of *Musa* species were recorded there (Pérez-vicente et al., 2014).

The banana was first described in the eighteenth century by the Swedish botanist, physician and zoologist Carl Linnaeus, who gave its name in 1750, simply adapted from the Arabic word for banana, "mauz" (Dan Koeppel, 2008). Botanist Linnaeus also translates the name of the yellow and sweet banana as *Musa sapientum* from the Latin term meaning "wise".

Musa, is genus name for banana and it falls into family Musaceae. The genus *Musa* embraces four sections, *Australimusa*, *Callimusa*, *Rhodochlamys* and *Eumusa* (Ploetz et al., 2007; Stover & Simmonds, 1987; Wardlaw, 1961). The majority of cultivated and edible bananas arose from the *Eumusa* section, being the biggest and geographically most ranging section of the genus (Stover & Simmonds, 1987). Almost all edible bananas derived from diploid species *M. acuminata* (A) and *M. balbisiana* (B) (Office of the Gene Technology Regulator, 2008). They are group into AA, AAA, AAB and

ABB genomic group depending on the relative participation of the genome in the genotype (Pillay et al., 2004).

2.2 The botany of banana

Banana plants are perennial monocotyledon, parthenocarpic (seedless) polyploids and classed as an arborescent herb (Price, 1995). It is a giant herbaceous flowering plant, with an apparent pseudostem that bends without breaking and composed of tightly packed leaves arrange in sheaths which rolled into a cylinder about 20 – 50 cm in diameter (Jones, 2000). There are three main part of the banana tree (Figure 2.1). The first part is the upperpart containing the midrib (the centre spine of the leaf), next is the pseudostem (made up of leaf sheath; the centre of the tree), and the last part is the corm. Phenotypically, wild species of banana are able to grow up to 15 meter with circumference of 2.5 meter. The morphology of the pseudostem varies between cultivars, especially in its size of length, disposition and pattern of Highland, sweet bananas and plantains. Highland as well as sweet banana's pseudostem mostly range from green to dark green with black spots, whereas plantains are yellowish green with brown spots. The underground corm also known as rhizome is the true stem. The meristem of the epical bud firstly gives rise to the leaves before it elongates up through the pseudostem and appear after 10 – 15 months of planting as a bulky terminal fluorescence (Pillay & Tripathi, 2007). As a monocotyledon plant, their root system is adventitious, arising from an organ rather than the root itself. Its roots spreading out laterally as far as 5.5 meter and form a dense mat mainly in the top 15 cm of soil.

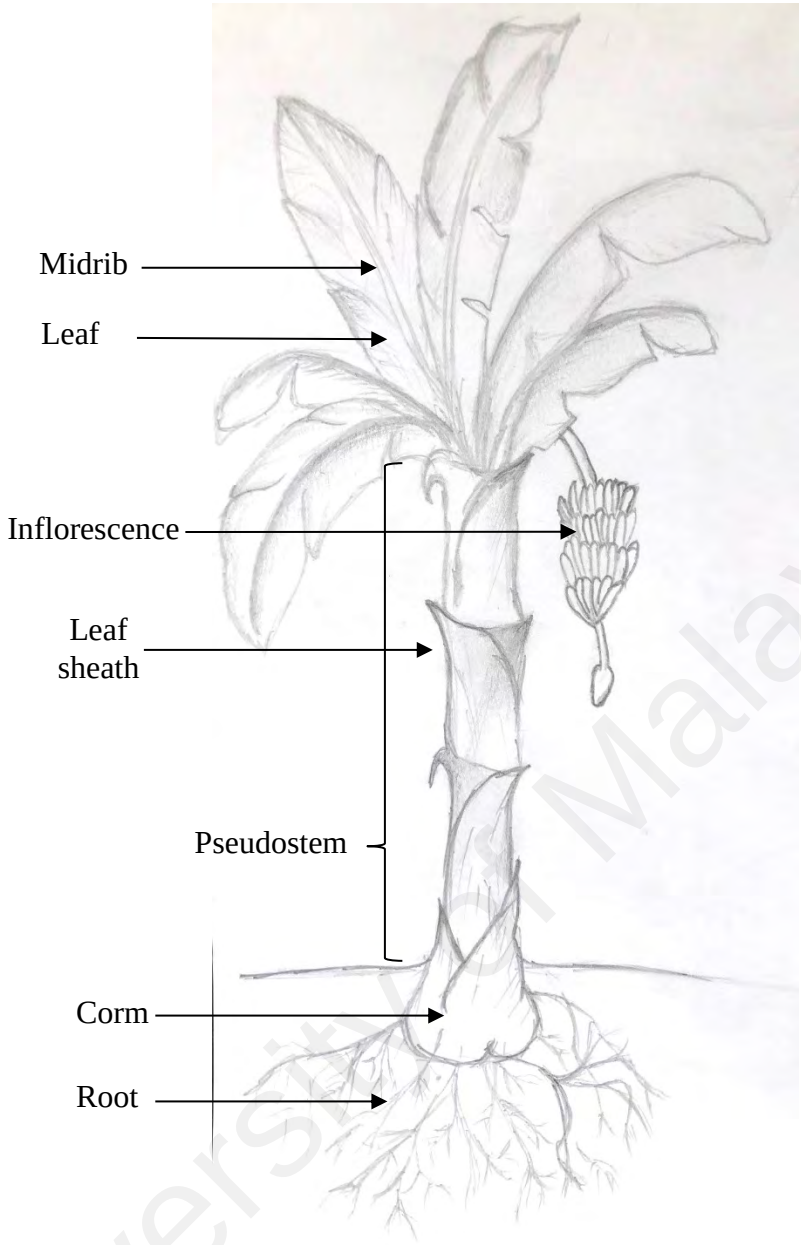


Figure 2.1: The anatomy of banana plant.

Banana can be grown in almost any kind of soil that is at least 60 cm in depth, has good supply of drainage, and not compacted (Stover & Simmonds, 1987). All Musa species grows best in open sun area provided moisture is not limited (Simmonds, 1962). Even though this plant can withstand shade up to 80%, it is recommended maximum of 50% of shade is most suitable. If the plants are sheltered too much, their apparent pseudostem gets thinner, leaf production and sucker will be reduced, fruiting will also be reduced and bunches formed will get smaller. The mat, also called stool will die if

they undergo deep shade (Ploetz et al., 2007; Simmonds, 1962). Burning up the banana plant on fire will never kill the plant as they able to recover by regrown again from the corm (Nelson et al., 2006).

Bananas are also able to withstand strong winds, which can twist and distort the crown. However, in extreme condition, the whole plants can be uprooting especially during heavy rains and hurricanes. Thus, in windy zones, dwarf varieties are excellent selections. They are characterizing by thick, petite pseudostem, compact structure, and small, wide leaves. These characteristics also serve other benefit of being easier to harvest with less green waste for disposal (Nelson et al., 2006).

Just like other crops, the yield and productivity of bananas is controlled by a number of biotic and abiotic stress factors that exist in the immediate environment of the banana plant (Heslop-Harrison & Schwarzacher, 2007). Among these, biotic stress is chiefly imparted by numerous diseases and pests, in which if it is present in that area above threshold level, it will stop the cultivation of banana plantation. The most destructive among the banana diseases are Fusarium wilt (Panama disease), leaf spot diseases (Black Sigatoka), Moko disease, fruit rots and infestations of viruses such as banana mosaic virus and banana bunchy top virus (Bakry et al., 2009).

2.3 Fusarium Wilt

2.3.1 Origin of Fusarium wilt disease

Fusarium wilt is caused by a soil borne fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*) and is one of the most destructive plant disease recorded in history (Moore et al., 1995; Ploetz & Pegg, 2000; Wardlaw, 1972). Fusarium wilt of banana was first recognized in 1876 at Eagle Farm, near Brisbane, Australia in the variety Sugar (AAB-Silk) by Dr. Joseph Bancroft (Pegg et al., 1996) but is now widely spread and exists in all major countries where bananas are grown, except those bordering the Mediterranean, Melanesia, Somalia, and a few islands in South Pacific (Ploetz, 1994; Stover & Simmonds, 1987).

After the incident in 1876, the outbreak of disease was next reported in banana plantations of 'Gros Michel' grown for export in Central America plantation during the year 1890 (Hwang & Ko, 2004). Although the first report of the disease was given by Dr Joseph Bancroft in 1876, the pathogen was successfully isolated by Smith for the first time from banana tissue sent to him from Cuba (Ploetz, 2005) who named it *Fusarium cubense* Smith. The first detailed description of the Fusarium wilt disease and the pathogen was reported by Ashby in 1913, while in 1919, Brandes validated pathogenicity conclusively debunked that *Fusarium oxysporum* that caused the Panama disease. Brandes was able to illustrate the disease symptoms in the paper with colour photograph, which were uncommon during that era (Ploetz, 2005).

In 1940, the name *Fusarium oxysporum* f.sp. *cubense* was proposed by Snyder and Hansen as they created special forms, formae speciales, in order to classify the pathogenic strain of *Fusarium oxysporum* that effected closely related host taxa (Ploetz, 2005). By the 1950s, the outbreak of the disease had reached such epidemic proportions that it was considered one of the most destructive plant diseases in recorded history

effecting the banana plants (Pegg et al., 1996). Gros Michel was a dominant cultivar in the commercial banana industry during that era.

From 1890 to the mid-1950s, Panama disease threatened about 40,000ha of banana plantation in Central and South America, threatening the very existence of the export trades thus causing severe yield loses in banana plantation industry (Pegg et al., 1996). According to the formal reports during that time, the epidemic caused loses of 22, 000 ha of Gros Michel in Republic of Panama, 13, 000ha in Costa Rica, 3 000 ha in Honduras and 2 200 ha in Guatemala (Ordonez et al., 2015; Ploetz, 2005). This significant pandemic was amongst the direst in horticultural history and resulted in the demise of the Gros Michel-based banana export production, caused by Panama disease.

The export banana industry was then saved by the introduction of the cultivar 'Cavendish' (AAA genome) which was resistant to *Fusarium oxysporum* f.sp. *cubense* (O'Donnell et al., 1998) after most of the Gros Michel plantations succumbed to Fusarium wilt. Epidemic of Panama disease did not stop there, as it later emerged in Cavendish banana in the late 1960s while the breeders were busy cultivating their Cavendishes, thus giving enough time for the Panama disease to evolve a new strain that was able to kill them off. The outbreaks in Cavendish subgroup was detected in the late 1960s in Taiwan and subsequently in South Queensland of Australia, Canary Island of Spain, South Africa (Ploetz, 1990) and Southeast Asia (O'Donnell et al., 1998). Emergence of the new strain of *Fusarium oxysporum* f. sp. *cubense* that was virulent towards Cavendish cultivar was identified in 1994 as new race, that is, race 4 in addition to the existing race 1, 2 and 3 (Su et al., 1986).

Until today, Race 4 has been the most destructive *Foc*, especially the tropical race 4 (TR4). From the time when the TR4 ruined the Cavendish banana industry in Taiwan, its outbreak in Southeast Asia region became more overwhelming with its spread in the

Chinese countryside of Guangdong, Fujian, Guangxi, and Yunnan as well as in Hainan Island. Since 1990s, the TR4 has also wiped out Cavendish plantations in Indonesia and Malaysia. The strain has also significantly affected the banana industry near Darwin in the Northern Territory of Australia. In the early 2000s, symptoms start to show in the Cavendish banana farms in Davao, Philippine in which it is currently threatening the entire national banana export trade (Molina et al., 2009). Since 2013, invasions of the TR4 strain from Southeast Asia were reported in Jordan (García-Bastidas et al., 2014), Pakistan, and Lebanon (Ordoñez et al., 2015). The attack of *Foc* TR4 were informally announced in Mozambique and Oman in the year of 2013. Outbreak of TR4 was initially noted on a farm in the Tully Valley, Far North Queensland, Australia on 3rd March, 2015 which detected on Cavendish banana plants (Cook et al., 2015). By now, the *Foc* TR4 may have affected up to approximately 100,000 hectares (Ordoñez et al., 2014), and will likely disseminate even more, either through infected plant material, contaminated soil, tools, footwear, flooding and inappropriate sanitation measures (Jones, 2000; Ploetz, 2005). This creates a huge concern that TR4 may eventually cause serious destruction due to the massive monoculture of these susceptible Cavendish bananas. This would threaten not only the export trade but also regional food provision and local economies if no countermeasures are established and applied to deal with this outbreak (Buddenhagen, 2009). At present, Fusarium wilt is still regarded as one of the most significant threats to banana production worldwide along with wheat rust and potato blight.

2.3.2 *Fusarium* species

The genus *Fusarium* comprises of a large number of species of filamentous fungi that cause plant disease and produce extremely dangerous secondary metabolites, known as *Fusarium* mycotoxin (Ma et al., 2010). Recent studies conducted by international community of plant pathologists, has ranked two *Fusarium* species, *F. graminearum* and *F. oxysporum* as fourth and fifth places respectively on the list of top 10 fungal plant pathogens according to its significance in term of its scientific and economic values (Dean et al., 2012). Other than plant, some *Fusarium* species may also cause disease towards human such as having an impaired immune system (neutropenia, i.e., very low neutrophils count) (Gupta & Ayyachamy, 2012).

Fusarium oxysporum (Fo) has been described as the most common species in this genus, with broadest range of hosts, reflecting remarkable genetic adaptability (Ma et al., 2010). It is also a remarkably diverse adaptable fungus that has been found in a wide range of climatic conditions as either beneficial saprophytes or endophytes. Among the pathogenic strains of *Fusarium* species, *F. oxysporum* is the most dangerous (Lal & Datta, 2012). *Fo* consisting of a number of pathogenic and non-pathogenic strains depending on the ability of the fungus to cause disease. Pathogenic strains of *F. oxysporum* colonize the roots and cause Fusarium wilt disease. However, non-pathogenic strains of *F. oxysporum* did not cause disease as they are known to infect and colonize the cambium tissue of banana roots only, but do not enter the xylem region (Sutherland, 2013).

2.3.3 The biology of *Fusarium oxysporum* f. sp. *cubense* (*Foc*)

Pathogenicity variability within the *Foc* has led us to its subdivision of specialized form (formae speciales) or races, differentiated by their ability to cause the symptoms on specific banana cultivars (Booth, 1971).

Most formae speciales are pathogenic to a single host crop, for example *F. oxysporum* f.sp *cubense* (*Foc*) infect banana, *F. oxysporum* f.sp. *vasinfectum* (*Fov*) infect cotton (*Gossypium hirsutum L.*), and *F. oxysporum* f.sp. *dianthi* (*Fod*) infect carnation (*Dianthus caryophyllus L.*). However, several formae speciales, can cause disease to more than one host, for instance *F. oxysporum* f.sp. *radicis-Iycopersici,* can cause disease on tomato and *Lycopersicon* species (Kistler, 1997). There are at least 150 formae speciales within *F. oxysporum* (Baayen et al., 2000) that can be further divided into races (Armstrong & Armstrong, 1981).

2.3.4 Physiological races and vegetative compatibility groups

A range of approaches is usually needed to characterize *F. oxysporum* f. sp. *cubense* isolates. According to virulence toward specific banana cultivars (Ploetz, 1994), the pathogen will be classified into one of the four races (i.e., races 1, 2, 3 or 4). A race is based upon the virulence of individuals in a formae speciales to a set of differential host cultivar (Correll, 1991). Race designation in *F. oxysporum* can be a simple subdivision with a single cultivar defining a single race, or a more complex subdivision where several cultivars are host to a single pathogenic race. Example of single-cultivar races are found in the tomato (*Solanum Iycopersicum L.*) while multiple-cultivar races are present in *Foc* where race 4 attacks Cavendish bananas as well as all cultivars that are attacked by races 1 and 2 (Fourie et al., 2009).

Race 1 strain pathogenic to cultivars like Gros Michel (AAA), Silk (AAB), Pomme (AAB), and Pisang Awak (ABB). Race 2 affect Bluggoe (ABB) known as cooking banana, all cultivars genetically related to it and some AAAA tetraploids. Race 3 attacks *Heliconia* spp. which is a close relative of banana. As for race 4, there are two strains co-existed, which are the tropical race 4 (TR4) strain and subtropical race 4 (ST4) strain. These types are identified by their ability to attack Cavendish bananas and all cultivars susceptible to race 1 and race 2 under tropical and subtropical climates respectively (Bentley et al., 1998; Gerlach et al., 2000; Ploetz, 1994; Ploetz, 1990; Su et al., 1986; Ploetz, 2000). ST4 attacks Cavendish bananas previously exposed to cold winter temperature and has been reported in South Africa, Australia, Taiwan and the Canary Island (Ploetz, 2006). TR4 infects Cavendish bananas in the tropical regions of Southeast Asia and Australia (Kistler et al., 1998; Ploetz, 1994).

Confusion in race structure of *Foc* often causes inaccuracy in describing strains of *Foc*. Results of race determination may be ambiguous because of environmental conditions (Stover & Buddenhagen, 1986). For this reason, *Foc* has also been categorized by the 'Vegetative Compatibility Groups' referred here as VCG as a means to categorize the pathogen. Conventionally, it relies on heterokaryon formation and may be determined with complementation test between auxotrophic nutritional mutants (Leslie, 1993; Puhalla, 1985). Puhalla (1985) is the first that developed an efficient technique to determine the compatibility, which use nitrate-non utilizing auxotrophic (*nit*) mutants being readily recovered and stable. In order to have a stable heterokaryon formation, two isolates must share a common allele at every *vic* locus (Correll, 1991). Thus, it could be expected that, the rest of the genomes of asexual species would also very similar for isolates in the same VCG (Leslie, 1993). This means that, a mutation detected at a single *vic* loci would place closely related individuals in different VCG (Bentley et al., 1998). Isolates in the same VCG also often share common biological,

physiological and pathological attributes (Caten & Jinks, 1966). Gordon et al., (1992) in his study revealed that isolates belongs to the same VCG all had the same mitochondrial DNA haplotype. Based on their data, they also conclude that, weak vegetative interaction may permit transfer of mitochondria between isolates in different VCG. Even though vegetative compatibility provides a clear measure of phenotypic relatedness, the technique does not measure the genetic relationship between VCGs and must be assessed by other means (Bentley et al., 1998; Fourie et al., 2009). The relatedness between VCG and race is even more complex, with a single race being associated with multiple VCGs (Katan & Primo, 1999).

In Malaysia, the VCG 01213/16 was widespread over western Peninsular and found on six cultivars affected by the pathogen. Details of VCGs and cultivars that they infect can be seen in Table 2.1. Mostert et al., (2017) also observed that a single isolate from the VCG complexes 0124/5 and *F. oxysporum* isolates are not compatible with known VCGs that they tested. There were also four other VCGs found in Malaysia but none of them from Malaysian Borneo. VCGs 01217 and 01218 were found in northern Peninsular Malaysia (Figure 2.2). VCG 0123 found in the northeast and northwest of Peninsular Malaysia, and VCG 0128 in the Kelantan area but cultivar information were not available.

They also found twelve isolates of *F. oxysporum* obtained from bananas in Malaysia which were not able to be identified. Two of these were VCG incompatible, while the other 10 isolates were not compatible to known VCG testers and they are Mas, Pisang Kapas, Pisang Abu Keling, Pisang Berangan, Pisang Awak, Pisang Rastali and Plantain.

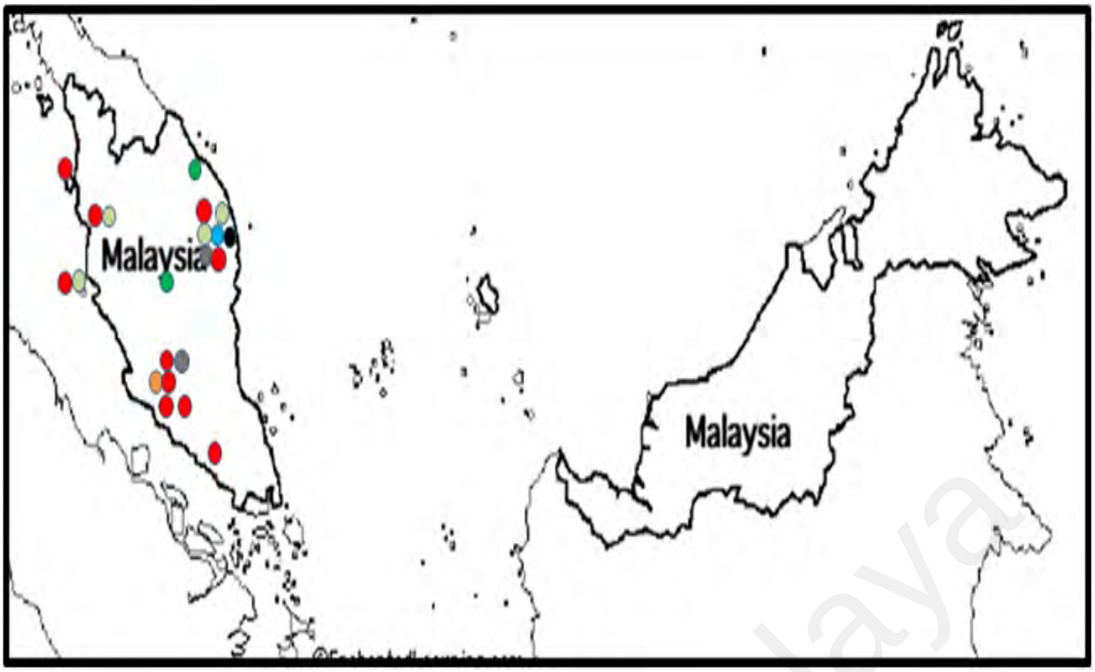


Figure 2.2: *Foc*'s VCG distribution around region of Malaysia. VCG 0121 is shown in light orange (●), VCG 0123 is shown in light green (●), VCG 0124/5 is shown in dark green (●), VCG 0128 is shown in blue (●), VCG 01213/16 is shown in red (●), VCG 01217 is shown in black (●) and VCG 01218 is shown in dark grey (●). Citation report graphic is derived from PLOS ONE, with permission from Mostert et al. (2017).

Table 2.1: Group, subgroup and cultivar of *Musa acuminata* with their relationship of vegetative compatibility groups (VCGs) and race for *Fusarium oxysporum* f. sp. *cubense* in Malaysia.

Group	Subgroup	Cultivar	VCG
AA	Sucrier	Pisang Mas	01213/16
AAA	Lakatan	Berangan	01213/16, 0121
AAB	Pisang Raja	Raja	01213/16
	Silk	Rasthali	0123, 01217
ABB	Pisang Awak	Awak	0123, 01213/16
	Bluggoe	Pisang Abu Keling	01213/16, 01218
Other	Other	Port Dickson	01213/16, 0128

The VCGs and races of *Foc* worldwide distribution are shown in Table 2.2. A total of 24 known vegetative compatibility groups (VCGs) have been recognized for *Foc*, where 21 of them are present in Australia and Asia (Bentley et al., 1995; Dita et al., 2010; Moore et al., 1993; Ploetz & Correll, 1988;Ploetz, 2005). Some VCGs are cross-compatible, thus it produce VCG complexes, such as VCGs 0120/15, 0124/5 and 01213/16. Even though the explanations of cross compatibility were unable to be described, it was believed that these VCGs were closely related to one another and represent a diverge population of the same VCG thus producing a sub-population. The sub-population of the VCG complexes was believed to have lost the capability to form a consistent heterokaryons (Ploetz, 1990).

The largest number of *Foc* VCGs is found in Indonesia and Malaysia, where *Foc* is thought to have originated. Still, the diversity of VCG in Asia appears to be distributed in discrete areas where it is influenced by the variety of banana grown and the prevailing climatic conditions. For example, in the sub-tropic area, VCG 0120 often effects Cavendish bananas, while in the tropics, VCGs 0121, 01213 and 01216 most commonly cause disease in Cavendish and also effecting other diploids and triploids banana (Jones, 2000).

Table 2.2: Grouping of *Foc* according to its respective VCG, race and geographical distribution.

No	VCG	Race	Distribution by country
1	0120	1, 4	South Africa, Australia, Brazil, Costa Rica, Honduras, Jamaica, Indonesia, Guadeloupe, Canary Islands, Malaysia, Taiwan.
2	0121	4	Indonesia, Malaysia, Taiwan.
3	0122	4	Philippines
4	0123	1	Philippines, Indonesia, Malaysia, Thailand, Taiwan, Vietnam.
5	0124	1,2	Australia, Burundi, Brazil, Cuba, USA, Honduras, India, Jamaica, Kenya, Malaysia, Malawi, Nicaragua, Philippines, Thailand, Uganda, Tanzania, Vietnam.
6	0125	1,2	Australia, Brazil, Honduras, India, Jamaica, Kenya, Malaysia, Malawi, Nicaragua, Philippines, Thailand, Uganda, Tanzania, Vietnam, Zaire.
7	0124/0125		Australia, Brazil, Cuba, EUA, Honduras, India, Indonesia, Jamaica, Kenya, Malaysia, Malawi, Nicaragua, Philippines, Thailand, Uganda, Vietnam.
8	0126	1	Honduras, Indonesia, Philippines.
9	0128	1,2	Australia, Comoros Islands, Cuba, Kenya, India, Thailand.
10	0129	4	Australia.
11	01210	1	Cuba, USA.
12	01211	4	Australia
13	01212	-	Kenya, Tanzania, Uganda.
14	01213	TR4	Australia, Indonesia, Malaysia.
15	01213/01216	TR4	Australia, Indonesia, Malaysia, and Papua New Guinea.
16	01214	2	Malawi.
17	01215	1,4	Costa Rica, Indonesia, Malaysia.
18	01216	TR4	Australia, Indonesia, Malaysia.
19	01217	-	Malaysia, Bangladesh.
20	01218	-	Indonesia, Malaysia, Philippines, Thailand.
21	01219	-	Indonesia
22	01220	4	Australia, India, Kenya, Thailand.
23	01221	-	Thailand.
24	01222		India, Bangladesh, Cambodia and Vietnam

2.3.5 Morphology of *Foc*

Fusarium oxysporum f. sp. *cubense* were traditionally classified as a Deuteromycete, member of the Elegans section of *Fusarium* (Fungi Imperfecti). A teleomorph (sexual stage) of *Fusarium oxysporum* has not been found, and the pathogen appears to rely solely on asexual mode of reproduction. The fungus produces three types of asexual spores; microconidia, macroconidia and chlamydospores for reproduction and dispersal (Leslie & Summerell, 2006).

Microconidia are a type of spores that are abundantly produced under all conditions including spores that have been produced inside the xylem vessel of infected plants. The macroconidia are found in dead plant tissue as well as in groups that look like sporodochia. The chlamydospores are round in shape, usually produced on older mycelium. The chlamydospores, also called resting spores are able to survive the longest in soils, generally in cold environments (Agrios, 2005). Figure 2.3 show details morphology of microconidia and macroconidia. Microconidia are usually one (no septate) or two celled (one septate) with oval to kidney-shaped (Figure 2.3A) and some are borne with false head (Figure 2.3B). The size dimension for microconidia usually in the range of 5-16 µm x 2.4-3.5 µm. Macroconidia are four to eight celled, sickle-shaped with thin walled and are delicate (Figure 2.3C). Basal cells are foot-shaped with attenuated or hook shaped at the apical cells, with usual dimension range of 27-55 µm x 3.3-5.5 µm (Fourie et al., 2011; Porter et al., 2015). Both micro and macroconidia are classified as short-lived spores while chlamydospores are classified as long-lived spores (Elliott, 2011). The chlamydospores are usually globose and are formed singly (7-11 µm) or some in pair in hyphae or conidia (Figure 2.3D). Some isolates like *Foc* with VCG 01214 do not produce this type of spores (Ploetz & Pegg, 2000) and in certain isolates, their development are slower (Pérez-vicente et al., 2014).

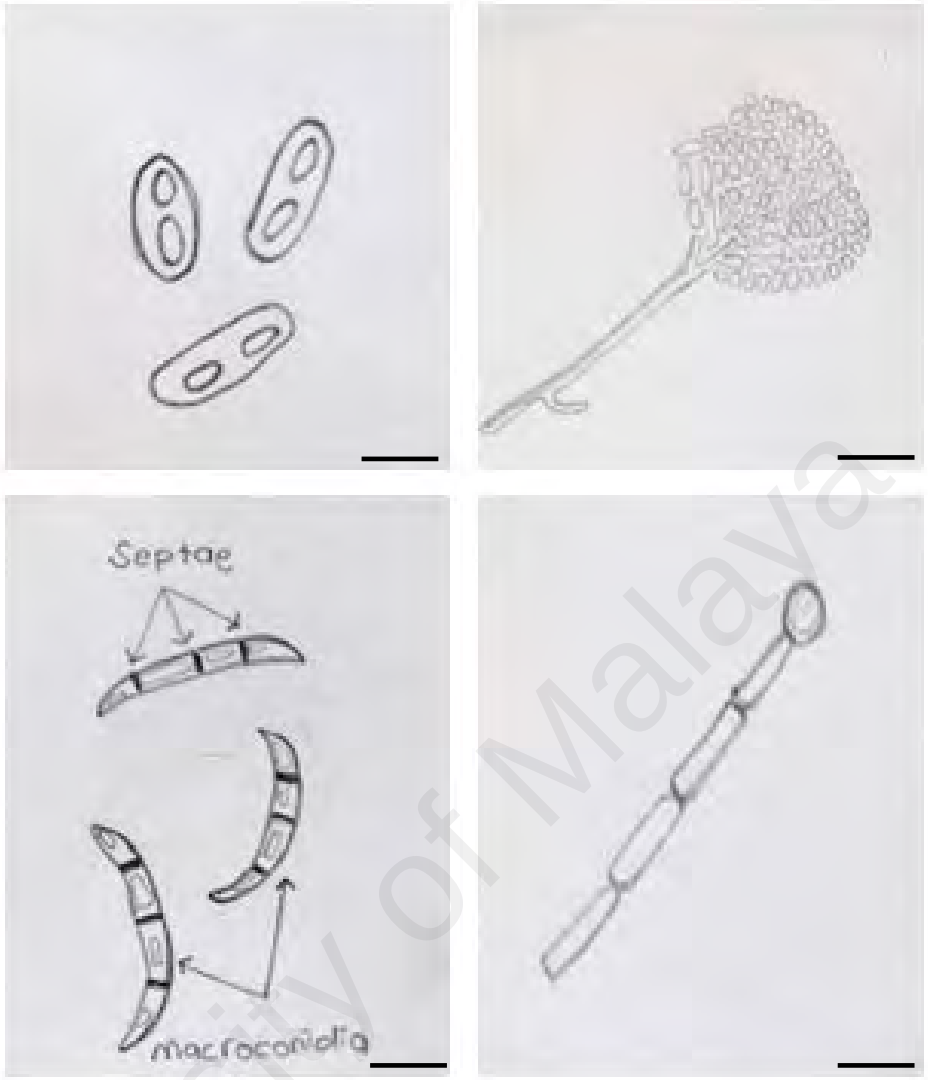


Figure 2.3: Asexual spores of *Fusarium* species. A) Microconidia with smaller oval shaped having no to one septate. B) Microconidia in false-head structure. C) Macroconidia with sickle-shaped and thin-walled. D) Single, terminal chlamydospores. Scale bar = 10 µm.

2.3.6 Life cycle of *Foc*

The life cycle of *Foc* (Figure 2.4) starts with a saprophytic stage where the fungus endures in soil as dormant spores called chlamydospores (Beckman & Roberts, 1995). Chlamydospores remain dormant and survive in the remains of decayed plant debris until stimulated to germinate by using nutrients that are released from extending roots of a variety of plants (Beckman & Roberts, 1995). Following germination, a thallus is produced from which conidia will start to form in 6-8 hours upon *Foc* infection, and chlamydospores in 2-3 days if conditions are favorable. Invasion of the roots is followed by the penetration towards the epidermal cells of a host or a non-host (Beckman & Roberts, 1995) and the development of a systemic vascular disease in host plants (Stover, 1970). In the progressive stages of the disease, the fungus grows out of the vascular system into adjacent parenchyma cells, proliferating and producing huge numbers of conidia and also chlamydospores. The pathogen survives in infected plant debris in the soil as saprophyte mycelium and in all its spore forms, but most commonly as chlamydospores in the cooler temperate regions (Agrios, 2005).

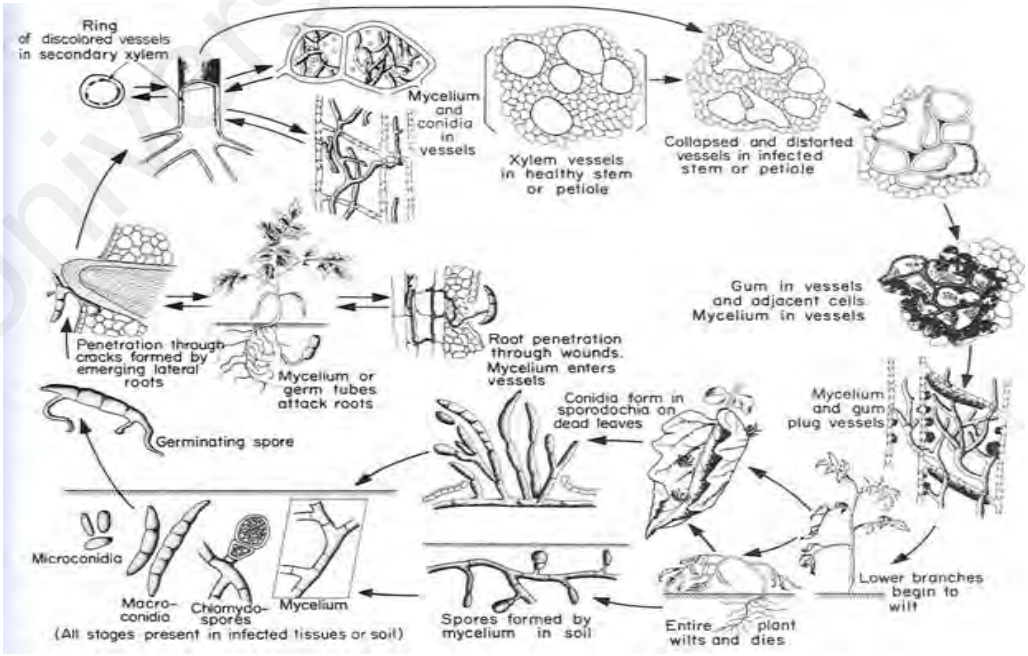


Figure 2.4: *F. oxysporum* life cycle in general. Citation report graphic is derived from Agronomie.info, with permission from Agrios, (2005).

Details life cycle of *Foc* in banana was shown in Figure 2.5 below. *Foc* is able to survive in the soil for up to 30 years as chlamydospores in infected debris or in the roots of alternative hosts. The fungus is also able to survive in the roots of symptomless alternative hosts such as close relative of the banana and several species of weeds and grasses under similar field condition (Moore et al., 1995).

The plant is often able to prevent infection from *Foc* by producing gels and tyloses (a resistance mechanism) to seal the infection. However, multiple infections may occur during a life of a plant and invariably one or more lead to its complete invasion. The virulence strain of *Foc* TR4 on 'Cavendish' cultivar suggest that the resistance mechanism was employed by the plant against this strain are not as effective as for STR4. This strain usually only cause serious losses in a plantation where the plants are under stress (Daly et al., 2006).

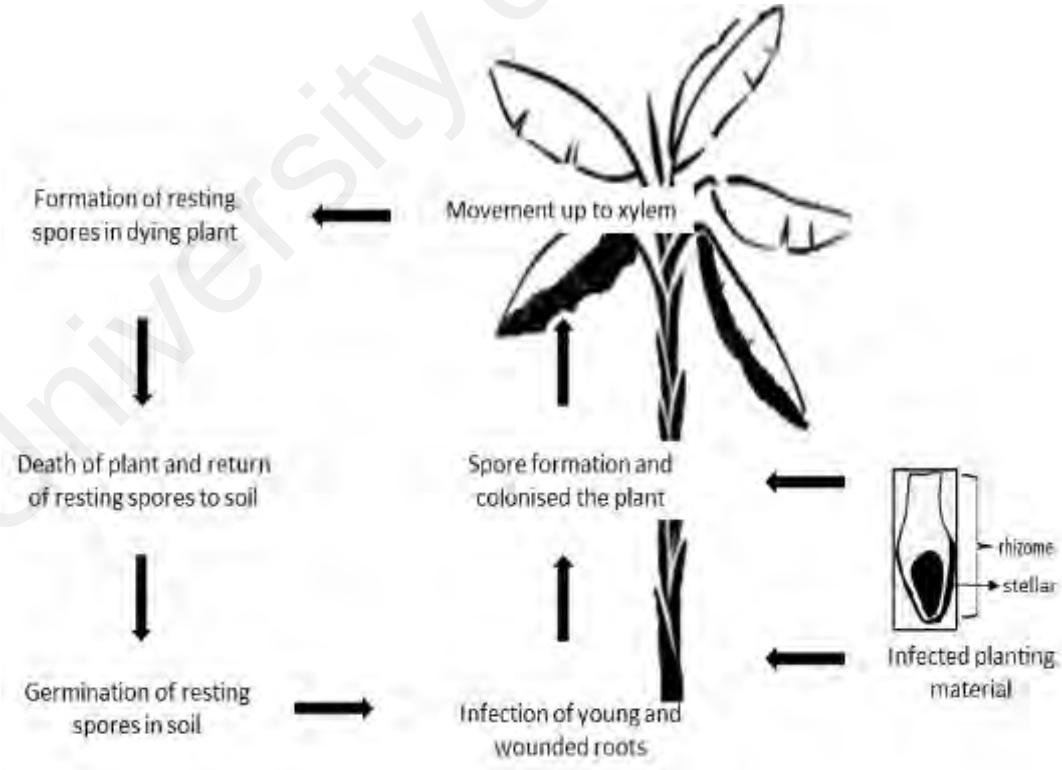


Figure 2.5: Disease initiation and life cycle of *Foc* in a banana plant.

2.3.7 Process of vascular infection by *Foc*

As a soil inhabitant, the pathogen is able to remain dormant in the absence of a host, mainly in the form of thick-walled chlamydospores or conidia (Smith, 2007) Once an area becomes infested with *F. oxysporum*, it usually remains there for many years (Agrios, 2005) until the establishment of the host occur. The ultrastructure of the infection progression has been well documented by a series of light and electron microscopy studies (Rodríguez-Gálvez & Mendgen, 1995) and most recently using green fluorescent protein (GFP) as a marker system (Di Pietro et al., 2001; Lagopodi et al., 2002).

Successful infection by *F. oxysporum* is a complex phenomenon that requires a series of highly regulated processes. These include adhesion, penetration, colonization and disease development. Each step is influenced by a broad range of integrated intrinsic and external factors, which in the end determine the pathogenicity.

A successful infection of *Foc* is achieved by adhesion of spores to the host. This process is necessary and it is normally achieved through the secretion of mucilage. A part from that, enzymes, lectins, and also hydrophobic and electrostatic forces also play a role in this process (Boucias et al., 1998). Site-specific binding may play an important role in anchoring the propagules at the root surface, after which other processes required for colonization can take place (Recorbet & Alabouvette, 1997).

After successful process of adhesion, penetration step will occur. Process of penetration is accomplished by a combination of different factors including fungal compounds, plant surface structures, activators or inhibitors of fungal spore germination, and germ tube formation by the host (Mendgen et al., 1996). The method of pathogens penetrating the roots may differ, but there are two distinct types. Some pathogenic hyphae adhere to the host young root tips and penetrate the epidermis

directly (Wen et al., 2009) whereas some enter indirectly through wounds (Lucas,2009). The most common sites of direct penetration are situated at the root tip of both tap roots and lateral roots (Lucas, 2009). The pathogen enters the apical region of the root where the endodermis is not fully differentiated and fungi are able to colonize and reach the developing protoxylem. *Fusarium oxysporum* has been found to penetrate the root cap and zone of elongation intercellular (Laith, 2017).

During the process of colonization, the mycelium invades rapidly, elongating the root cortex until it reaches the xylem and xylem parenchyma of susceptible plants (Roberts & Boothroyd, 1984). At this point of infection, the fungus switches themselves to a highly peculiar mode of infection, in order to remain exclusively within the xylem vessels, using them as paths to rapidly colonize the host. This is mainly accomplished by the production of microconidia, which are detached and carried upward in the sap stream. The microconidia eventually germinate and the mycelium penetrates the upper wall of the vessels, producing more microconidia in the next vessel.

After all of the process above occur, development of disease will take place. During disease development, characteristic wilt symptoms appear as a result of severe water stress, mainly due to clogging of the vessels. Wilting is most likely caused by a combination of pathogen activities, such as the accumulation of fungal mycelium, spores, or polysaccharides (Roberts & Boothroyd, 1984). The xylem may be further plugged by toxin production as the result of host defense responses, including production of gels, gums, tyloses and vessel crushing by proliferation of adjacent parenchyma cells (Beckman, 1987). Once vascular tissues turn brown as the effect of colonization by the fungus, it is seen as the result of vascular wilt disease thus providing a diagnostic symptom for the disease to the farmers (Roberts & Boothroyd, 1984). As a result of the blockage and breakdown of xylem vessels, external symptoms to the host

such as leaf wilting and yellowing of the leaf appear before the plant eventually dies. As long as the plant is alive, the vascular wilt fungus remains strictly limited to the xylem tissues and a few surrounding cells. Only when the infected plant is killed by the disease, the fungus invade the parenchyma tissue and sporulates profusely on the surface of the dead plant (Ploetz & Pegg, 2000).

2.3.8 Dissemination of the disease

The pathogen is spread most commonly through vegetative propagation of infected rhizome (Moore et al., 1995; Su et al., 1986). They also can be spread as spores in soil through running water (Jones, 2000), and soil adhering through farm tools and machinery (Moore et al., 1995; Ploetz & Pegg, 2000). Since water is one of the relevant paths of disease dissemination, attention must be taken in the water from surface run-off and irrigation as it can spread the pathogen in a short distance, while natural disaster such as floods may cause long distance dissemination of the pathogen (Moore et al., 1995; Ploetz & Pegg, 2000; Su et al., 1986).

2.3.9 Fusarium wilt symptom

As mentioned earlier, symptoms of Fusarium wilt disease can be observed internally and externally on the infected plants. The characteristic of internal symptoms are characterized by reddish to dark brown discoloration of the host's vascular system.

The first internal symptoms occur in the roots and corms, and then progress to the pseudostem (Moore et al., 1995; Ploetz & Pegg, 2000; Wardlaw, 1961). Through a transversal cut of the plants, more intense discoloration may be observed on the

outermost sheaths of the pseudostem in direction to the innermost or young leaf sheaths (Figure 2.6 and 2.7C) (Pérez-vicente et al., 2014).

The earliest external symptoms of Fusarium wilt in banana are yellowing of the oldest leaf reaching progressively to the youngest leaves by a vivid band along the margin and subsequently spreading inwards toward the mid ribs (Figure 2.7A). Sometimes, the typical and conspicuous external symptom may be confused with potassium deficiency, especially in dry and cold climate condition. The yellowing then advances till it causes wilting and collapse of the leaves at the petiole, hanging down to form a "skirt" of dead leaves around the pseudostem (Moore et al., 1995; Ploetz & Pegg, 2000; Su et al., 1986; Wardlaw, 1961). At this stage, a distinct red-brown discoloration of vascular tissue can be clearly seen when the pseudostem is cut (Figure 2.7B) (Davis, 2005; Wardlaw, 1961). Once the plant dies, it takes about 1-2 months before the infected plants to start decaying and topple (Ploetz & Pegg, 2000).

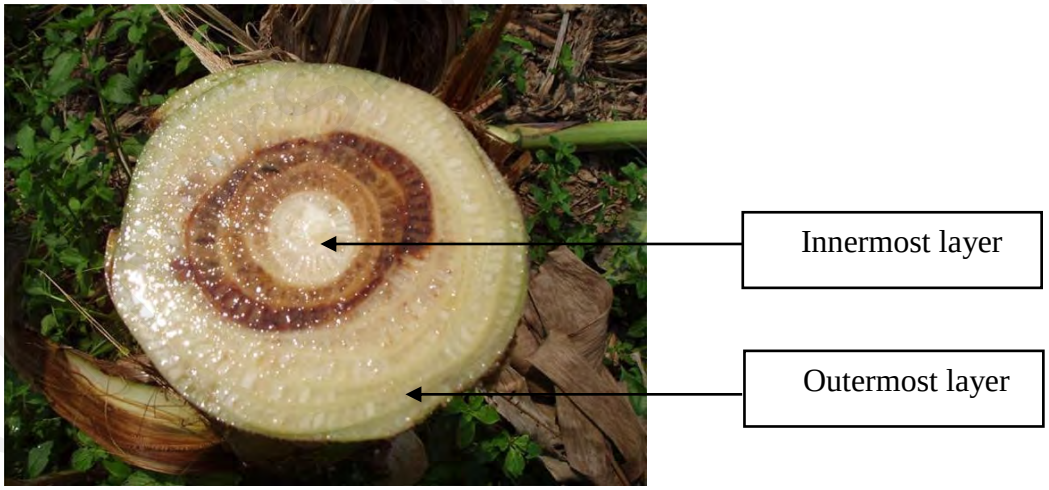


Figure 2.6: Transversal cut of pseudostem of infected banana showing internal discoloration of pseudostem as the result of Fusarium wilt disease. Arrow shows the innermost and outermost layer of the pseudostem. More intense of discoloration was observed at the outermost layer of pseudostem. Citation report graphic is derived from Creative Commons Attribution 3.0 Australia, with permission from Liberato et al. (2006).

Figure 2.7: Fusarium wilt disease symptoms. A) External symptoms (yellowing and buckling of leaves) of Fusarium wilt disease observed in infected plants. B) Brown discoloration in the pseudostem of infected plant. C) Brown discoloration in the corm of infected plant by Fusarium wilt disease. Citation report graphics is derived from IntechOpen, with permission from Thangavelu et al. (2012).

2.3.10 Control and management strategies of Fusarium wilt

Fusarium oxysporum and its many *formae speciales* affect a wide variety of hosts, causing its management to differ among pathosystem. In general, chemical treatments, biological control, cultural control and resistant cultivars can be included in an integrated disease management strategy to control the dissemination of the disease.

2.3.10.1 Chemical control

Chemical control measures of the diseases can be divided into four groups, namely fungicides, surface sterilants, fumigants and plant activators. Chemical control can also be coupled together with commercial biological product containing microbes and fungicides. Elmer and McGovern, (2004) found out the combination of beneficial microbes with fungicides able to give higher degree of inhibition of Fusarium wilt in cyclamen. Fungicides is one of potential chemical which can be consider in integrated disease management programme to control Fusarium wilt disease in banana (Gullino et al., 2000). The demethylation-inhibiting fungicides (DMI) such as prochloraz, propiconazole and combination of cyproconazole and propiconazole, were the most effective one to inhibit the growth of *Foc* through in vitro and glasshouse. These fungicides are known as “azole group” which works by inhibiting the demethylation step in the biosynthesis of a compound call as sterol, which is important in the fungal cell walls. However, all these fungicides could only be used by means of soil drenches or root dripping, as it is proved that application through stem injection was ineffective (Nel et al., 2007).

Surface sterilant is also one of the essential components in disease management program to control the introduction and dissemination of Fusarium wilt in fields (Meldrum et al., 2013; Moore et al., 1999). Nowadays, sterilant such as

copperoxycloride, chlorine bleach and methylated spirits have been replaced by more environmentally friendly products to ensure low environmental toxicity such as 'Farmcleanse ®' and Sporekill® (Moore et al., 1999; Nel et al., 2007). Those sterilants were used to disinfect all equipment that were used to transport soil, farming tools and machinery (Meldrum et al., 2013).

Soil fumigation has been a widely used strategy to exterminate soil pathogens. This technology involves introducing gas-forming chemicals such as carbon disulfide, methyl bromide, or chloropicrin into soil to kill target pathogens (Ben-Yephet et al., 1994; Li et al., 2014). However, these methods cause undesirable side effects. It kills beneficial organisms, cause contamination towards groundwater, and the toxic produced by these chemicals had cause this technique less reliable method for disease management strategy (Ben-Yephet et al., 1994; Maloy, 2005).

2.3.10.2 Biological control

Biological control is an environmentally sound and effective means of reducing the pathogenicity of soil pathogens by using endophytic antagonistic microorganisms to provide an alternative to control the disease dissemination (Pocasangre, 2000). Many groups of microorganisms have been proposed as having a role in protecting host plants from vascular wilt pathogens. Of these, non-pathogenic *Fusarium oxysporum* and *Trichoderma* spp. was proved to provide promising results as biological control of Fusarium wilt (Nawangsih et al., 2011; Pal & Brian, 2006). Apart from that, recent studies conducted by Tan et al. (2015) proved that endophytic bacteria strain ITBB B5-1, isolated from the rubber tree, and identified as *Serratia marcescens* has demonstrated biocontrol activity to the wilt pathogen under both greenhouse and field. In the future, biological combination between endophytic bacteria with commercial pesticides may

lead to synergistic effects towards eradicating the disease. Soils suppressive to soil borne pathogens have also been identified worldwide and attributed mainly to suppress soil borne pathogen (Schlatter et al., 2017). This type of soil are naturally suppressive to diseases induced by soil borne plant pathogens thus allow production of host plant although the pathogen are there (Alabouvette, 1999).

2.3.10.3 Quarantine

While all of the above mentioned strategies are being improved from time to time, the most important method of Fusarium wilt control is quarantine. Quarantine as a measure to control the spread of the pathogen, which involves limiting the movement of pathogens in infected planting material between countries or between fields. Prohibition and eradication must be stressed in the forefront to ensure the control of disease dissemination (Dita et al., 2010; Molina et al., 2009; Moore et al., 1995; Wardlaw, 1961).

2.3.10.4 Resistant cultivar

Out of all types of disease management and control strategies, the utilization of resistant cultivar of banana against *Fusarium oxysporum* is the most effective management tool (Moore et al., 1995; H. J. Su et al., 1986). The current problem that we are facing now is that, there are no cultivars available, which offer the organoleptic quality presented in the 'Cavendish' and 'Gros Michel' subgroup to combat race 4 invasions. Micro propagated plant materials are the most promising source of clean material, as they are also free from bacteria, nematodes, and other fungal pathogens. Hence, plantlets ought to be utilized at whatever point is conceivable (Moore et al., 1995; Ploetz & Pegg, 2000; Su et al., 1986; Wardlaw, 1961). An understanding of how

plants defend themselves on a molecular level is a crucial step towards generating resistant plants, where resistance is not readily available in closely related species and wild progenitors (Rejeb et al., 2014).

2.4 Assessment of Fusarium wilt resistance

It is well known that the use of resistant cultivars is the only sustainable way of managing Fusarium wilt in banana (Collinge et al., 2010; Punja, 2001). Fusarium wilt resistance cultivar can be produced by genetic engineering technology. However, the ability to differentiate between susceptible and resistant plants is very much essential. The use of bioassays approach also is being used to relate the level of antimicrobial enzymes or stress related compounds on field evaluations in naturally infected fields, as the laboratory studies given varying outcomes (Strömstedt et al., 2013). The inconsistency of laboratory results has been attributed to the fact that plants are sometimes subjected to unrealistic level of inoculum density inside the soil. Laboratory assays usually depends on the use of tissue culture generated plants and their responses towards infection are fluctuated, thus the results produced were not valid. This is because they lack natural defenses properties due to the elimination of some endophytes during micro propagation stage. In addition to this, it also causes them to be highly susceptible when introduced to the field (Smith et al., 1998). Field assessments are more consistent yet they are tedious and costly and this required improvement of a quick, reliable and consistent small plant bioassay.

2.4.1 Infection assays

Several available screening methods have been used to screen resistance level of tissue culture banana towards Fusarium wilt disease. Majority methods are either a closed pot system or hydroponic solution system. It is also important to take into consideration some factors such as the age of plantlets, the inoculation method, presence or absence of endophytes and fungal virulence or spore type used. Those factors may affect the susceptibility of plants and therefore producing inconsistent results (De Beer Zaag et al., 2001; Smith et al., 2008; Subramaniam et al., 2006; Wu et al., 2010). Studies by Smith et al. (2008) demonstrated that plantlets that are 8 weeks-old with height range between 10 – 15 cm were most suitable to provide consistent results for infection assay compared to those plants that are less than 10 cm in the pot system. However, plantlets that are grown by using hydroponics system seem to have much smaller in size and height (Groenewald et al., 2006).

To summarize, there are a few different inoculation techniques that were used to performed bioassay experiment; wounded roots dipped into a suspension of conidial, potting mix inoculated with millet grains that were colonized by *Foc*, and potting mix or liquid media medium inoculated with a suspension of *Foc*'s conidia.

In spore suspension protocol for inoculation method, wounded plant roots were immersed in spores suspended in liquid media at a various concentration. The control group was treated in the same manner but the plantlets were inoculated with distilled water. Immediately after inoculation, the plants were transferred to the pots and placed in a greenhouse (Morpurgo et al., 2010).

In protocols using substrate such as millet grains, the fungus was allowed to colonize the substrate first for a few days. Later, the colonized substrates were applied directly onto the plant roots. Method of using substrate colonized by inoculum with millet or

sorghum was found to be better than using spore suspensions as it allows spore multiplication and survival which is the main significant event for infection (Smith et al., 2008).

The last method of bioassay is using potting mix or liquid media medium inoculated with a suspension of *Foc*'s conidia. In order to perform this kind of approaches, pots were drenched with 40 ml conidial suspension. A 1 cm layer vermiculite was then placed on the surface of the potting mix (Smith et al., 2008).

All studies mostly evaluated the disease progression on 7 to 8 weeks post inoculation for inoculation using pot system (Matsumoto et al., 1995; Smith et al., 2008) and 6 weeks for hydroponics system (Groenewald et al., 2006; Van Den Berg et al., 2007). All of the bioassay experiments were performed in greenhouse. Various concentration of spore suspensions were used with different time of incubation and had been compile as shown in Table 2.3 below.

The culture media from which the spores for *Foc* inoculation are grown can also determine the efficiency of infection. This can be observed from the studies by Smith et al. (2008), where he demonstrated that spore suspension from carnation leaf agar (CLA) was found to be more infectious than those from potato dextrose agar (PDA). The results were obtain as the CLA was able to provide growth for all the different types of conidial while PDA favored the growth of microconidia and a few macroconidia only (Smith et al., 2008). To date the most reliable culture media used by most researchers group is PDA compared to CLA. Method using CLA seems too complicated as the carnation leaf should be sterilize using gamma-irradiation.

Table 2.3: Different parameters involved in *Foc* bioassay studies.

Reference	Inoculum concentration	Inoculation period	Plantlet age & size	Disease evaluation	Post-challenge analysis	Disposal protocols	Remarks
(Mak, Mohamed, Liew, & Ho, 2004)	10^6 spores/ml	2 h	2-mo-plantlets, 10-15cm height	Based on Brake et al., (1995); Javed et al., (2004)	NA	Contaminated water was treated with sodium hypochlorite	Double-tray set up
(Thangavelu & Mustaffa , 2012)	10g of inoculated sand-maize with *Foc* inoculum per pot.	NA	3-mo-plantlets	NA	Peroxidase activity, PAL activity, Chitinase assay & β-1,3-glucanase activity	NA	*Foc* colonized on sand-maize medium for 15 days.
(Li et al., 2012)	5000 conidia/g of soil	NA	4-5 leaves, 30 cm height	NA	Transcriptome analysis	NA	Suspension was added to planting medium
(Javed et al., 2004)	10^6 spores/ml	3 h	4-5 weeks	Brake et al., (1995)	Random amplified polymorphic DNA (RAPD)	Mohamed et al., (2001)	Double-tray set up
(Subramaniam et al., 2006)	$2x10^4$, $2x10^6$ & $2x10^8$ spores/ml	1 h. Additional 1 ml of different spore concentration	1 week	Leaf chlorosis	Hydrogen peroxide, phenylalanine ammonia lyase, chitinase and β-1,3-glucanase assays	NA	Double container

Table 2.3, continued.

(Mahdavi, Sariah, & Maziah, 2012)	2x10^8 spores/ml,	1 h. Additional 1 ml of different spore concentration was applied	2-mo	Leaf chlorosis	NA	NA	NA
(J. Paul et al., 2011)	10^5 spores/ml	48 h	2 week	Based on Smith et al., (2008)	TUNEL assay	NA	In-vitro
	2 x 10^8 spores	In soil mix	2 mo		NA		Small bioassay (ex-vivo)
(Mohandas et al., 2013)	2.8x 10^6 spores/ml	In 150g sterilized sorghum grain	2 mo, 7-8 leaf stage	Based on (a) Leaves: Nasir et al., (2003); (b) Corms: Orjeda, (1998)	Western blot, ELISA	NA	NA
(Lijia Guo et al., 2014)	10^6 conidia/ml	48 h	NA	NA	Transcriptome, qPCR	NA	NA
(Pérez-vicente et al., 2014)	10^6 spores/ml	30 min	45 d 15-25 cm height	External & internal evaluation	NA	NA	Double pot

2.4.2 Disease validation of virulence studies

It is important to determine the disease symptoms that strongly indicate the results obtained showing whether it is associated with susceptibility or resistance of the host plants for disease assessment. Infection by *Foc* in banana plants starts in the site of root hairs elongation zones. Usually, both susceptible and resistant cultivars are affected. As mentioned earlier, once the root hairs are invaded by the pathogen, the hyphae network of the pathogen will start to propagate through the epidermal cell. The hyphae invades dermis and cortex cells via pores (Chunyu et al., 2011). Different phenomena was observed in resistant plants, where complete occlusion of the vessels close to the hyphae occurs (Nasir et al., 2003). For those susceptible plants, delay in formation of tyloses and quick breakdown of gels was observed (Mace et al., 2012). The association of rhizome infection and wilting symptoms of the leaves in confirming resistance or susceptibility of the host is not always correlated (Paul et al., 2011). Thus in most reports, many author confirms that rhizome discoloration is the best indicator of disease validation of virulence studies (Nasir et al., 2003).

2.5 Response in banana to Fusarium wilt

Plants responses to attack by pathogen are either to hinder it completely (resistant plants) or to minimize its effect (tolerant plants). Sometimes the pathogen succeeds in infecting plants, leading to disease (susceptible plants) (Swarupa et al., 2014). Level of resistance and susceptibility in plant-pathogen systems rely on the constitutive and induced defense response functions from the host. Host plants have developed an innate defense system against the invasion of pathogens and at the same times, the pathogens have evolved themselves to suppress the plant defense system.

2.5.1 Recognition of pathogen

Detection of invasion by potential invader either pathogens or non-pathogens is a prerequisite for an efficient defense response to be activated. Normally, the plant cell surface has special receptors called pattern-recognition receptors (PRR) which functions to detect the pathogen, via pathogen/microbe-associated molecular patterns (PAMPs/MAMPs). This detection of the pathogen by the receptor will then activate basal resistance or PAMP-triggered immunity (PTI). This will happen in both non-host and host plants (Zipfel et al., 2006). PTI then initiates some other intracellular responses associated with plant defense mechanism. Response including ion fluxes across the membrane, reactive oxygen species (ROS) and ethylene production, mitogen-activated protein kinase cascades, plant cell wall reinforcement at the site of infection, and closing of stomata will collectively contribute to PTI. However, if this first line of innate immune response in plants is successfully been invaded by the pathogen, the plant's resistance (*R*) genes will later recognize the invasion from the effectors (Avr) of the pathogen to activate effector-triggered immunity (ETI) (Wu et al., 2014).

ETI is generally similar to PTI, but the differences rely on the degree of specificity. ETI is more specific and faster than PTI as it correlates with gene-for gene defense response (Jones & Dangl, 2006). ETI response encompasses defense signaling events, the expression of pathogenesis-related (*PR*) genes, systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants (Durrant & Dong, 2004). Regardless of how pathogens are detected, either through effectors or PAMPs, the plant's defense system is controlled by several hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxins, gibberellins, abscisic acid (ABA), cytokinins, brassinosteroids, and peptide hormones (Bari & Jones, 2009).

2.5.2 Structural defense of plant

As mentioned earlier, the plant surface contains the first line of defense that the pathogens must penetrate before they can cause infection towards the plant that they attack (Agrios, 2005; Swain, 1977). Therefore, resistance from penetration by epidermal cells from pathogen invasion is an important component of defense mechanism in plants (McDowell & Dangl, 2000). Natural plants defense mechanisms are often present in the plant even before the pathogen comes in contact with the plant, and resistance can be attributed by several factors including barriers such as cell walls strengthened by lignin (Wuyts et al., 2005). Natural structural defenses of host plants can also be triggered by both pathogens and non-pathogens (Dangl & McDowell, 2006). During induction of structural plant defense, plant cell walls are fortified at the sites of penetration, a phenomenon known as the cell wall apposition (CWA) (Hardham et al., 2007).

Other physical mechanism of structural defense in plants include the occlusion of colonized vessels by gums, gels, and tyloses which form to prevent further ingress of the invading pathogens (Schmelzer, 2002). Tyloses formation, also called as xylem occlusion is considered as a resistance reason against the invasion from *Foc* in resistant banana cultivars (Raman et al., 2016). The resistance is due to an inhibition of the upward spread of the fungus, thus limiting the growth of the pathogen (Beckman, 2000). Tylose formation has been successfully found 2 days after inoculation of a resistant banana cultivar within vessel luminal of roots (VanderMolen et al., 1987). For susceptible plants where the gels are short-lived, tylose formation is delayed or is not formed at all, thereby allowing conidia to spread ahead of the vascular occlusion (Beckman, 2000).

2.5.3 An overview of Pathogenesis-related (PR) 10 proteins

Pathogenesis-related (PR) protein are ubiquitous protein found in monocot and dicot plants. PR-10 proteins are involve in plant growth and defence mechanism but their molecular function is still unknown. Many studies were conducted and show that it involve in many process such as enzymatic processes, synthesis of secondary metabolites, antimicrobial process, storage, membrane binding and other hydrophobic ligand binding processes (Jain & Kumar, 2015). PR protein act as ribonucleotide binding protein and involve in virus resistance through attacking the viral RNAs (Huh & Paek, 2013). Several studies show that PR-10 proteins have several function but there is no general function in common. It seems that the post translational modification of it offer important specificity of its target RNAs (Park et al., 2004).

Recently, Baharum et al. (2018) investigated the function of PR-10 gene in response towards *Foc* against *Musa acuminata* cv. Berangan. They integrate the full length cDNA sequences of *PR-10* gene from wild banana into a commercial cultivar, "Berangan" and name it as *MaPR-10* via co-cultivation of transformed *Agrobacterium tumefaciens* and embryonic cell suspension. They found out that, line-19 Berangan harboring *MaPR-10* has shown delayed disease response during infection by *Foc*.

2.5.4 *Phenylalanine Ammonia-Lyase (PAL)* gene

Phenylalanine ammonia-lyase ((PAL) is an enzyme which is found in many plants that act upon catalysation of metabolic pathway of phenylpropanoid compounds. A type of phenylpropanoid metabolites known as phenylphenalenons, have been found in banana and it involved in resistance mechanism against pathogens attack (Alvarez et al., 2013). Other studies by Wang et al. (2007) propose that PAL plays an important role in promoting defense mechanism against abiotic stress. In many higher plants, PAL play an

important role in general defense mechanism towards attack by pathogens (Mur et al., 1996).

In banana, a number of partial cDNA sequences of PAL including two partial isoforms (MaPAL1 and MaPAL2) are known to be related in postharvest chilling tolerance in banana (Wang et al., 2007). Alvarez et al. (2013) emphasize that differential expression of PAL in banana varying between different cultivars.

2.6 Detection of banana pathogens: conventional and molecular approaches

Prompt detection and monitoring of these pathogens once they attack is crucial for the development of effective management plans. The lack of quick, precise, and efficient means by which plant pathogens can be detected and monitored is a main drawback in integrated disease management strategies.

2.6.1 Conventional or traditional approach

Conventionally, *F. oxysporum* f. sp. *cubense* detection in banana relies on the isolation of fungal pathogen from infected plant parts or soil. Process of identification mainly relies on culture-based morphological technique (Capote et al., 2012). However, this system is laborious and time consuming which makes it difficult to process large sample sizes in a short period of time (Lin et al., 2009). It often provides inconsistent outcomes and requires significant information of fungal taxonomy (Jurado et al., 2006). Furthermore, this technique is less sensitive and less specific, because *F. oxysporum* f. sp *cubense* is morphologically similar to other fungal pathogens when grown on a nutrient medium.

The identification of *Fusarium* species is mainly based on distinctive characters of the shapes and sizes of macro and microconidia, presence or absence of chlamydospores as well as appearances of its colony, pigmentations and growth rates on agar media (Leslie & Summerell, 2006).

Process of identification will be based on predicted work and therefore sometimes misdiagnosed (Naroei & Salari, 2015). Microscopically, it is often not easy to detect a specific fungus in root tissues and in soil that shows similar morphological pattern (Goud & Termorshuizen, 2003). These limitations have led to the development of molecular approaches as a research tool with improved accuracy and consistency (Capote et al., 2012).

2.6.2 Molecular method for *Fusarium* identification

The principal cornerstone to the control of Fusarium wilt is early detection and accurate identification of plant pathogen. In plants showing Fusarium wilt symptoms, detection can relatively be easy when one is qualified in disease analysis and plant pathogen isolation process. Conversely, for asymptomatic plant, the detection of pathogens in materials can be very challenging particularly when limited pathogen propagules are present, therefore sensitive techniques capable of detecting small amounts of pathogens is important (Capote et al., 2012).

The basic technique for molecular detection consists of a tissue sample taken from an individual from which DNA is extracted. Extracted genomic DNA will then serve as a reference model after which the genes are amplified and sequenced. The resulting sequence will then serve as an identification tag for the species from which the respective individual was derived. Molecular detection methods especially using nucleotide

amplification with polymerase chain reaction (PCR) are relatively faster, sensitive, highly specific, and accurate and results can be interpreted by personnel without taxonomic skills (Jurado et al., 2006). One of the most significant molecular techniques for an efficient PCR based pathogen detection is the development of species specific primers (Lin et al., 2010). The intergenic spacer (IGS) and internal transcribed spacer (ITS) regions of the ribosomal RNA genes also possess characteristics which allow identification of plant pathogens using molecular tools technique (Bao et al., 2002; Edel et al., 2000; Singh et al., 2006; Wu et al., 2002).

In recent years, there has been massive improvement in the development of molecular biological tools and technologies (Beckman, 1987). Each new revolutionary method can be used as a tool to study variation amongst fungal isolates, and hence provide significant data on genetic relationships, taxonomy, population structure and epidemiology of fungal species. The advancements of this technology allow fast, accurate detection and quantification of plant pathogen with more quicker and accurate detection (Paplomatas, 2004, 2006). Molecular methods such as PCR, RT-qPCR, RAPD, RFLP, SSR, ITS and rDNA marker are being used to distinguish between closely related species of fungal with few morphological characteristic differences within a species(Lal & Datta, 2012). The information resulting from such experiments could be used to improve disease management strategies by allowing more rational decisions to be made.

2.6.2.1 Polymerase Chain Reaction (PCR)

The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi (Bridge, 2002; Louie et al., 2000). Unlike fungal culture systems used, together with the generally low sensitivity and slow growth of many fungi, PCR application does not require the presence

of viable organisms for implementation. PCR technology has therefore improved the detection and may be performed with very small amounts of biological material (Abd-Elsalam et al., 2003).

Nowadays, PCR base detection method for pathogen detection is categorize as the most reliable, rapid and specific diagnostic method that can be used for TR4 detection. Those advantages allow farmers to monitor disease progression in the field. PCR assays have been used successfully in the past for the detection and identification of economically important of various *Fusarium* species (Doohan et al., 1998; Klemsdal & Elen, 2006; Wilson et al., 2004). Molecular tools have been used for pathogen detection by using the advancement of species specific primers. By using this emerging technique, fragments of *F. oxysporum* f. sp. c*ubense* can be amplified as molecular markers for identification and detection of *F. oxysporum* f.sp. *cubense* tropical race 4 (Dita et al., 2010). A set of specific primers have been used to analyze extracted DNA sample of pathogen and detection results were obtained in a short period of time compared to traditional isolation which is time consuming (Dita et al., 2010; Edel et al., 2000). The detection limit limits of conventional PCR assay was up to 100 pg of DNA. Thus, samples with DNA concentration lower than those are not suitable to be assayed using this method. However, real-time assay showed more sensitivity and was able to detect samples at DNA concentrations of 5 pg (Priyanka et al., 2015).

2.6.2.2 Reverse-Transcriptase quantitative PCR (RT-qPCR)

Quantitative Real-time PCR is a PCR-base technique that has become an extensively applied technique as it enables quantitative analyses of gene expression (Jozefczuk & Adjaye, 2011). This technique has appeared as a powerful and widely used approach as it can detect and quantify very small amounts of specific nucleic acid sequences in a

sample. It is also a good model system as it allows researchers to measure responses to experimental stimuli thus providing data to gain insight towards possible modifications in protein level and function (Valasek & Repa, 2005). RT-qPCR have been employed since 20 years ago in research to quantify level of pathogen during plant infection (Lamar et al., 1995). The fact that reverse-transcriptase PCR is highly sensitivity, makes it well-suited for studying pathogen growth during infection, especially as a reliable method for assessment of disease severity assay (Gachon & Saindrenan, 2004).

2.6.2.3 Advantages of Real-time PCR

Real-time PCR represents a technological leap forward that has opened up novel and powerful applications for researchers throughout the world and hold a number of benefits over other available methods to quantify gene expression (Wong & Medrano, 2005). Wang and Brown, (1990) in his studies showed that Real-time PCR assay was 10 thousand fold times more sensitive than RNase protection assay. Real-time PCR also was 1000 fold time more sensitive compared to dot blot hybridization (Malinen et al., 2003). The most powerful feature of Real-time PCR analysis is that it is able to detect a single copy of a specific transcript (Palmer et al., 2003). A part from that, real-time PCR is also reliable in detecting expression of gene differences as low as 23% between samples that are tested (Gentle et al., 2001). Studies by Schmittgen et al. (2000) demonstrated that real-time PCR was able to detect gene expression that have lower coefficient of variation where those using SYBR Green as lowest as 14.2% and TaqMan at 24%. With all of these benefits, it's shown that real-time PCR can be relatively high-throughput assay with proper equipment used with it. Real-time PCR is also able to distinguish among messenger RNAs (mRNAs) with almost identical sequences, thus this shows that less

RNA template are required compared to other gene expression assays available (Wong & Medrano, 2005).

2.6.2.4 Disadvantages of Real-time PCR

The only major disadvantage of real-time PCR is that, to perform analysis using this approach, it required equipment and reagents to run it properly. Unfortunately, both equipment and reagents for real-time PCR are expensive. Besides that, properties of extremely high sensitive assay causes this approach to need a proper experimental design and expertise to understand normalization technique to make sure accurate data and results of their studies are obtained (Wong & Medrano, 2005).

2.6.2.5 Other approaches.

(a) ***Universal Primed-PCR (UP-PCR)***

Universal primed PCR (UP-PCR) is a PCR fingerprinting technique, which amplifies specific target of DNA sequences by using species-specific primers (Paplomatas, 2004). The UP-PCR technique is an analogue to the randomly amplified polymorphic DNA (RAPD) technique. The technique consists of DNA amplification using single universal random primer with approximately 16 to 21 nucleotides, which are unique to the species tested. The reactions are carried out using relatively high annealing temperatures (55°C) in this marker system and results in highly reproducible amplicon from single organisms (Abd-Elsalam et al., 2003).

UP-PCR approach has been applied successfully to identify fungal pathogens populations, including *Fusarium* species (Abd-Elsalam et al., 2003; Bulat, Mironenko, & Zholkevich, 1995). This method has proved its applicability in various aspects of

mycology. Generally, this approach involves study of genome assemblies, species identification, analysis of population and diversity in species level , validating genetic similarity at intra- and inter-species level, and identify UP-PCR markers at diverse taxonomic levels (Abd-Elsalam et al., 2003; Yli-Mattila et al., 2004).

(b) ***Isozymes Technology***

Isozymes (also called as isoenzymes) are homologous forms of enzymes, which found in plant, animal and insect systematics and were later revised for use in phytopathogenic fungi taxonomy (Mfcales et al., 1993). Isozymes usually catalyze the same chemical reaction but it's amino acid sequences are slightly different (Latner, 1970; Mfcales et al., 1993).

Isozyme variation assay was originally applied to fungi whose morphological characteristics showed high levels of variation with species that are overlapping between one another. Although this assay could deliver sufficient levels of polymorphic loci, their usage in the fungal plant pathogens study has been restricted. This is due to a reason that they are subject to environmental stimuli which are able to cause polymorphisms, thus it cannot reflect evolutionary events (Mfcales et al., 1993; Micales et al., 1986). One of the drawbacks of isozyme analysis is that relatively large numbers of isolates and enzyme systems must be present, in order to extract sufficient enzymes for analysis (Zambino & Harrington, 1989), compared with what is required for immunological or PCR assay.

2.7 *Foc* genome

As mentioned previously, identification of *Fusarium* species is not complete just by study their morphology, because different cultural conditions will cause same species to vary (Doohan et al., 1998). The knowledge of their genome will definitely support their morphological characterization and value added to their molecular study. As for the *Foc* genome of Race 1 and Race 4, it was made known to the scientific community in 2014 (Guo et al., 2014).

2.7.1 Genome sequencing and general features

A group of research conducted by Guo et al. (2014) had performed a sequencing analysis of *Foc* isolates Race 1 (*Foc*R1) and Race 4 (*Foc*R4). Based on their research, they found out that the total assembled genome size of *Foc*R1 and *Foc*R4 were 47.84 Mb and 53.12 Mb respectively. They also found out that, the assembled size of both *Foc* isolates resembled *Foc* tropical race 4 strain II5 with genome size of 46.55 Mb, which was reported by Broad institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/). They predicted that *Foc*R1 and *Foc*R4 are having 17,462 and 18,065 coding genes, respectively. These coding capacities are similar to other ascomycetes such as *Foc* strain II5 and *Fol* strain 4287 with numbers of coding genes of 16,634 and 20, 925, respectively (Ma et al., 2010).

Guo et al. (2014) explains that the 5 Mb difference in the genome size of *Foc*R1 and *Foc*R4 is probably due to the fact that they construct more libraries with large inserts and they got more mate pair information for *Foc*R4. The advantages of having more mate pair information in *Foc*R4 is that they able to connect the contigs into scaffolds but more gaps were introduced. Both *Foc*R1 and *Foc*R4 coding region was approximate to 53.50% of

the total genome with 2.82 exons per gene with average of the exon length is 480 bp in size. Details of *Foc*R1 and *Foc*R4 genomes were shown in Table 2.4 below.

Table 2.4: Features of the *Foc* race 1 (*Foc*1) and race 4 (*Foc*4) genomes.

Features	*Foc* 1	*Foc* 4
Genome size (bp)	47, 838, 384	53, 119, 146
Coverage (fold)	106x	132x
G+C content (%)	47.98	48.05
Exon number	49, 212	50, 991
Exon length (bp)	22, 414, 389	23, 357, 730
Total annotations	15, 692	16, 288

2.7.2 Gene families and phylogenetic relationship of some sequenced *Fusaria*

Guo et al. (2014) revealed that 17,196 (98.5%) of *Foc*R1 genes were assigned into 12,366 gene families while 17,785 (98.3%) of *Foc*R4 genes were assigned into 12,365 genes families in total. They also create a phylogenetic tree between the two sequenced *Foc* isolates (*Foc*R1 amd *Foc*R4) and *Fol*. The result of the analysis revealed a close relationship between the sequenced samples thus, this suggesting that they might evolved from a common ancestor (Figure 2.8).

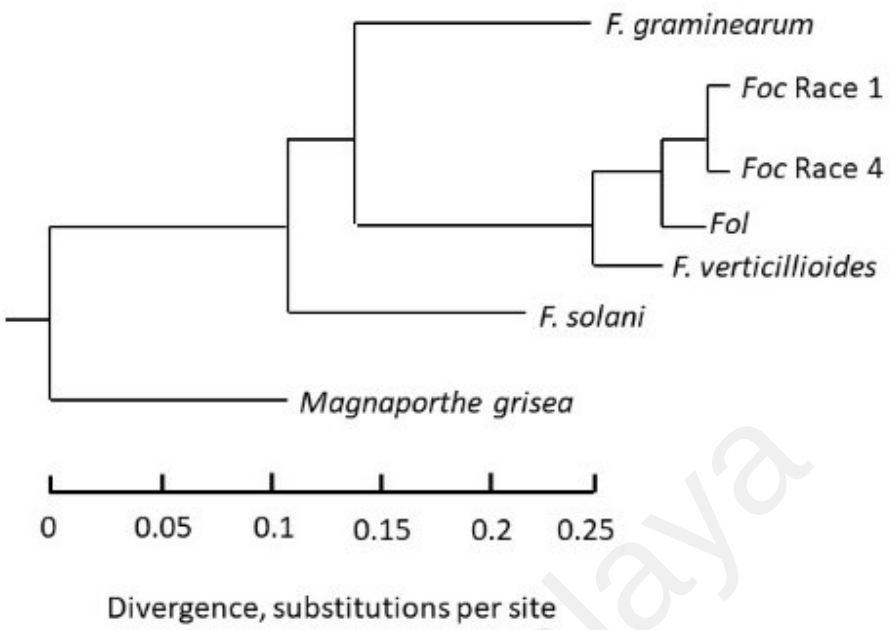


Figure 2.8: Phylogenomic relationships of both *Foc* isolates and other five sequenced fungi.

2.7.3 Virulence associated genes

A total of 347 and 348 putative virulence associated genes (*VAG*s) were identified in *Foc*R1 and *Foc*R4, respectively. A part from that, 15 *VAG*s have successfully characterized from *Fo* as shown in Table 2.5 below. These data demonstrating conservation in *VAG*s and pathogenesis between the diverse formae speciales of *Fo* species. A number of *Foc* orthologs were also found in other pathogenic fungi that infect cereal species such as *F. graminearum*, *Magnaporthe oryzae* and *Ustilago maydis* (Amey et al., 2003; Collemare et al., 2008). A part from that, there are many *Foc* orthologs were founds in animal pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans*. These findings could be clarified the causes of *Foc* having lack infection structure such as appressoria during host penetration process, which is similar to both animal pathogen mentioned earlier (Fassler & West, 2011; Roldán-Arjona et al., 1999).

Table 2.5: Characterized Virulence Associated Genes (*VAG*s) in *Fo* species.

No	VAGs	Characterized by
1	*ARG1*	Namiki et al., (2001)
2	*CHS2*	Martín-Udíroz, Madrid, & Roncero, (2004)
3	*CHSV*	Madrid, Di Pietro, & Roncero, (2003)
4	*CHS7*	Martín-Udíroz et al., (2004)
5	*FMK1*	Di Pietro et al., (2001)
6	*FGA1*	Jain et al., (2002)
7	*FGA2*	Jain et al., (2005)
8	*FGB1*	Jain et al., (2003)
9	*FOW1*	Inoue, Namiki, & Tsuge, (2002)
10	*FOW2*	Imazaki et al., (2007)
11	*FoSNF1*	Ospina-Giraldo, Mullins, & Kang, (2003)
12	*FRP1*	Duyvesteijn et al., (2005)
13	*GAS1*	Caracuel et al., (2005)
14	*PacC*	Caracuel et al., (2003)
15	*SIX1*	Rep et al., (2004)

2.7.4 Secreted protein

Both *Foc* encode for a total of approximately 1,300 secreted proteins (SPs) with 1,298 for *Foc*R1 and 1342 for *Foc*R4, which accounting for about 7.4% of the total predicted proteins. These numbers are a bit fewer than those recorded in *Fol* (1,541). (Guo et al., 2014). Studies on *Fol*, the causal agent of Fusarium wilt in tomato, have interpreted the roles of some SPs in pathogenicity of the *Fol*-tomato interaction. The secreted-in-xylem (*SIX*) proteins were first reported in the xylem sap of *Fol*-tomato pathosystem (Rep et al.,

2002). Three *SIX* protein, *SIX1, SIX3* and *SIX4* function as either *Avr* protein (effector) involved in the incompatible interaction or they could interact with the tomato genes (*I/I-1/I-2/I-3*) to induce resistance (Houterman et al., 2009; Lievens et al., 2009; Rep et al., 2004). Based from the information provided by Rep et al. (2002) and Guo et al. (2014) assembles data of *Foc*R1 and *Foc*R4, they discovered that three orthologs of *SIX1* interspersed in *Foc*R4 genome (known as *Six1a* - *Six1c*, Table 2.6), while in *Foc*R1, there is only one copy if *SIX1* was found. Besides, *Foc*R4 has one copy of *SIX2*, *SIX6* and *SIX8*, while *Foc*R1 only has one copy of *SIX6*. These data however, differ from the earlier study on *SIX6* genes using hybridization analysis and PCR where only one copy of *SIX1*, *SIX7* and *SIX8* genes were detected in another *Foc*R4 isolates from Australia (Meldrum et al., 2012).

Table 2.6: The orthologs of *SIX*-genes in *Foc* Race 1 and *Foc* Race 4 designated here as *Foc*1 and *Foc*4.

SIX genes	*Foc* race 1		*Foc* race 4	
	0h of post inoculation	48h of post inoculation	0h of post inoculation	48h of post inoculation
SIX1a	5.75	39.16	6.27	12.77
SIX1b	-	-	0.49	0
SIX1c	-	-	50.71	113.08
SIX2	-	-	NA	NA
SIX6	14.32	13.31	243.71	192.59
SIX8	-	-	0.16	0.63

Guo et al. (2014) also performed expression analysis of both *Foc* isolates. They found out that, the expression of the three copies of *SIX1* from *Foc*R4 and one copy from *Foc*R1 was detectable on both vegetative growth stage and 48 hours of post inoculation to banana

(Table 2.6). This result revealaed that the *SIX1* gene were functioned at both stages, and additional copies in *Foc*R4 may contribute to higher pathogenicity towards banana. This outcome was consistant with the result obtain from Does et al. (2008), where they also revealed that *SIX1* was induced immediately upon penetration of the root cortex in tomato. Furthermore, the other genes present in *Foc*R4 (*SIX2* and *SIX8*) that are absent in *Foc*R1, may be because these genes may have roles in infection of Cavendish banana 'Brazil' and may contribute to the broader host range of *Foc*R4 (Guo et al., 2014).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Fungal isolates were supplied by Prof. Baharuddin Salleh of Institute of Plant Pathology and Mycology Laboratory, School of Biological Science, University of Science Malaysia (USM), Malaysia and maintained as pure culture on Water Agar medium at the PhytoMycology Laboratory, University of Malaya. The fungi were originally isolated from the inner stem of an infected *M. acuminata* collected from various regions in Malaysia.

Our *Foc* sample for fieldwork study is from 'Hot-spots' in farmers' plantation located in Kelantan. Whilst, disease-free tissue culture-derived banana plantlets, *Musa acuminata* cv. 'Berangan' for pathogenicity test were purchased from Plant Biotechnology Unit (PBIU), University of Malaya.

3.1.1 Fungal isolates

A total of 42 fungal isolates mentioned in section 3.1 above and is listed in details in Table 3.1. These fungal isolates that formed the basis of this study were collected from infected *Musa acuminata* from different location in Malaysia as describe in Table 3.1. Starter cultures were prepared by transferring isolates from the water agar (WA) slants onto Petri dishes of full strength Difco^{TM} PDA (BD, France) supplemented with 1.2 ml of streptomycin sulphate for every 240 ml of media (Sigma Aldrich, Steinheim, Germany). All isolates were cultured for 7 to 14 days at 25°C upon identification. The isolates were confirmed to be *Fusarium* after observing colonial characteristics, microscopic features and molecular analysis.

Table 3.1: Fungal isolates details used in this study.

No	Isolate No	Banana's variety	Part of plant	Location
1.	235	Banana var. Wild	N/A	Grik, Perak
2.	236	Banana var. Wild	N/A	Grik, Perak
3.	239	Banana var. Wild	N/A	Grik, Perak
4.	240	Banana var. Wild	N/A	Grik, Perak
5.	241	Banana var. Wild	N/A	Grik, Perak
6.	243	Banana var. Wild	N/A	Grik, Perak
7.	255	Banana var. Wild	N/A	Grik, Perak
8.	523	Banana var. Wild	Crown rot fruit	Teluk Kumbar, Penang
9.	1454	Banana var. Raja	root	Trong, Perak
10.	1462	Banana var. Awak	N/A	Trong, Perak
11.	2279	Banana var. Emas	N/A	Changkat Jering, Perak
12.	2280	Banana var. Awak	N/A	Sri Iskandar, Perak
13.	2281	Banana var. Awak	N/A	Bota Kanan, Perak.
14.	2282	Banana var. Berangan	N/A	Titi Gantung, Perak
15.	2284	Banana var. Kapas	N/A	Klang, Selangor
16.	2288	Banana var. Raja Abu	N/A	Serkam, Melaka
17.	2290	Banana var. Awak	N/A	Lakluk, Terengganu
18.	2291	Banana var. Berangan	N/A	Kota Bharu, Kelantan
19.	2293	Banana var. Abu	N/A	Kota Bharu, Kelantan
20.	2294	Banana var. Berangan	N/A	Tumpat, Kelantan
21.	2295	Banana var. Abu	N/A	Lenggong, Perak
22.	2296	Banana var. Berangan	N/A	Kuala Kangsar, Perak
23.	2305	Banana var. Awak Masam	N/A	Kubang Semang, Penang
24.	2306	Banana var. Awak	N/A	Sri Iskandar, Perak
25.	2307	Banana var. Emas	N/A	Klang, Selangor

Table 3.1, continued.

26.	2327	Banana	stem	JTP, Johor
27.	2328	Banana	stem	JTP, Johor
28.	2330	Banana	stem	JTP, Johor
29.	2331	Banana	rhizome	JTP, Johor
30.	2332	Banana	rhizome	JTP, Johor
31.	2460	Banana var. Lilin	N/A	Serdang, Selangor
32.	2461	Banana var. Kelat Raja	N/A	Serdang, Selangor
33.	2462	Banana var. Lakatan Philippines	N/A	Serdang, Selangor
34.	2468	Banana var. Awak B	N/A	Serdang, Selangor
35.	2469	Banana var. Mas	N/A	Serdang, Selangor
36.	2471	Banana var. William Honduras	N/A	Serdang, Selangor
37.	2472	Banana var. Montel	N/A	Serdang, Selangor
38.	9886	Banana	root	Mr.Banana's Company, Johor
39.	9887	Banana	root	Mr.Banana's Company, Johor
40.	9888	Banana var. Cavendish	Inner part of stem	Kuala Terengganu
41.	C1 HIR	Banana var. Cavendish	Inner part of stem	Kuala Terengganu
42.	10201	Banana var. Cavendish	Inner part of stem	Kuala Terengganu

3.2 Methods

3.2.1 Research Methodologies

The research was conducted at the High Impact Research-CEBAR Facility, University of Malaya, Kuala Lumpur. The research was divided into 3 parts. The first part of the research is screening and characterizing all 42 collection of the isolates through morphological characteristics and molecular analysis.

In understanding the current status of the Fusarium Wilt Disease in Malaysia, it is crucial for smallholder's banana plantations to estimate their potential losses. The information permits the detection of disease-free zones and can be used to avoid the entrance of new strains where the fungus has limitedly diversified (Moore et al. 1999) through fast and precise detection through molecular analysis of the soil sample. The second part of this study, the occurrence and incidence of Fusarium Wilt was examined for study areas dubbed 'Hot-spots' in farmers' plantation located in Kelantan through collaboration with Dr. Fatimah from University Malaysia Kelantan (UMK).

Later, our last part of this research is to carry out pathogenicity test by bioassay experiment. Pathogenicity tests were performed on the disease-free tissue culture-derived banana plantlets, *Musa acuminata* cv. 'Berangan'. Acclimatized 2 month old *Musa acuminata* cv. 'Berangan' were treated with Race 1 and Race 4 of *Fusarium oxysporum* f. sp. *cubense* isolates using root-dipping approach. The plants were kept in a controlled greenhouse environment and observed for the development of the disease. Scoring for any development of external and internal disease symptoms was done by the end of the 5th week of post-inoculation. The pathogen was re-isolated from representative diseased plant roots infected with *Fusarium oysporum* f. sp. *cubense* to perform the molecular analysis. Protocols and procedures, which were significantly altered from their published practice, are designated in every section used. While precise conditions about these

techniques are described within the corresponding chapters. The flowchart of the research that was conducted is illustrated in Figure 3.1 below.

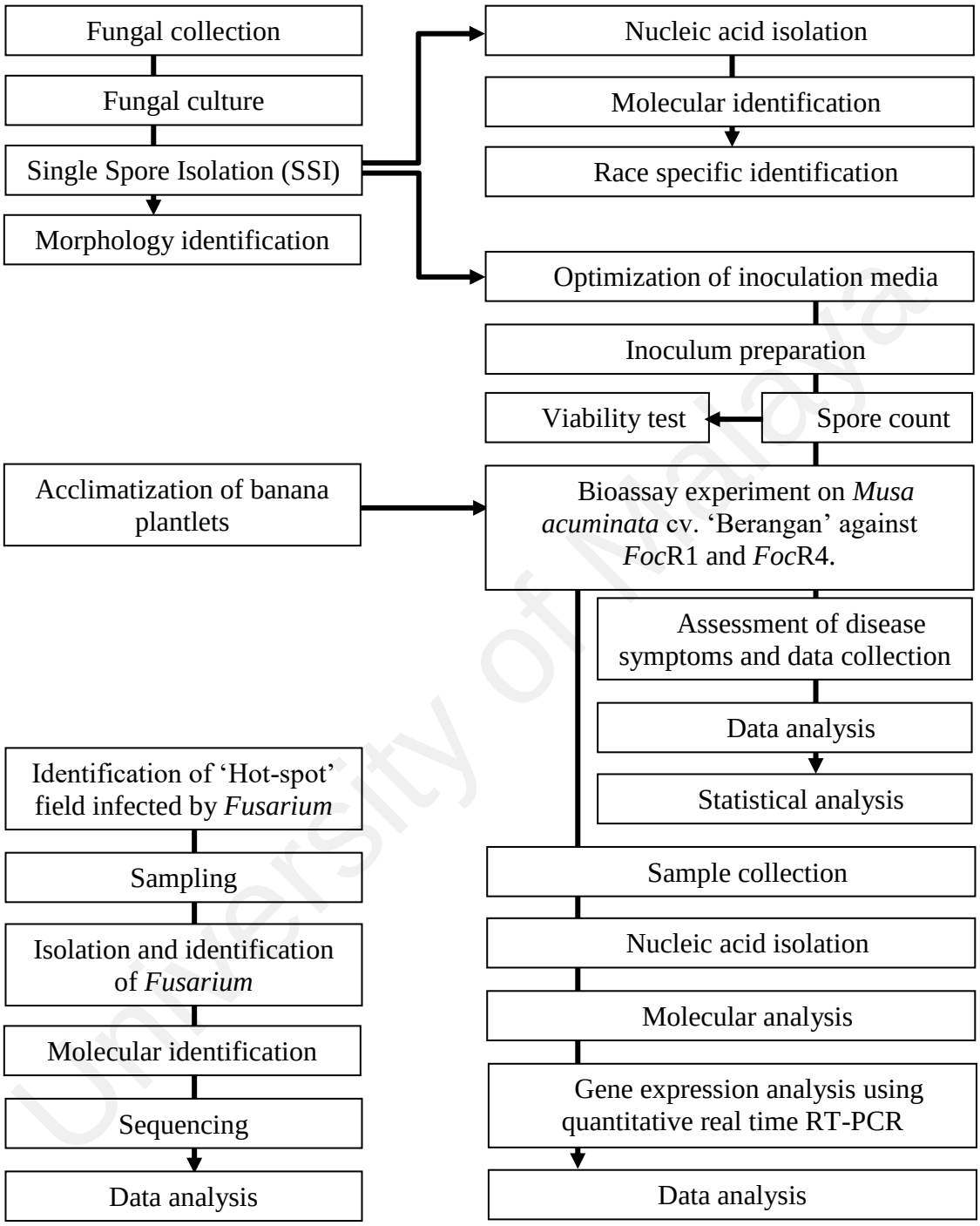


Figure 3.1: Project overview.

3.2.2 Sterilization

All glassware, micropipette tips, microcentrifuge tubes, PCR tubes, Falcon tubes, stock solutions and buffers for DNA and RNA experiments were autoclaved at 121°C at 15 psi for 20 and 45 minutes respectively. While, the media culture for fungal growth was autoclaved at 121°C at 15 psi for 20 min.

3.2.3 Pure culture of *Fusarium oxysporum* f.sp. *cubense*

Pure cultures of *Fusarium* were obtained by using the Single Spore Isolation (SSI) technique (Leslie & Summerell, 2006). The technique was also complemented with the aseptic procedure. All steps were carried out inside a laminar flow hood in order to ensure a controlled and sterile condition. An inoculation loop was sterilized by flaming it over a spirit methyl lamp until the wire becomes red-hot.

A small chunk of starter cultures from the WA slants containing *Foc* was scraped out and transferred onto a fresh PDA (Difco ™, BD, French) which was added with 1.2 ml of streptomycin antibiotic (Sigma-Aldrich, U.S.A) (for every 240 ml of media. The plates were then incubated at room temperature (RT) (27±°C) for 3 to 5 days in the dark. Every PDA (Difco™, BD, France) plate was labelled with an initial, date and the name of *Foc* isolate. The initial PDA (Difco™, BD, France) plate was labelled as PDA1. Healthy mycelia appeared on the agar surface after 3 to 5 days of incubation. A sterile loop was used to scrap out the mycelia from PDA1. The mycelia were suspended into a 1.5 ml micro centrifuge tube containing 1.5 ml of sterile distilled water and mixed by inverting up and down for several times. One loop-full of the dispersed mycilia were streaked onto a fresh plate of PDA (Difco™, BD, France) (assign as PDA2) using the quadrant-streaking technique. The PDA2 plate was incubated at RT for 2 days with the cap on the upside position. Single isolated colony was selected and transferred onto a new PDA

(Difco^{TM},BD, France) (assign as PDA 3). PDA (Difco^{TM}, BD, France) 3 was incubated at RT (27±2℃) for another 3 to 5 days to obtain a pure culture. A detailed diagram of the SSI method is shown in Figure 3.2 below. Modification was made where instead of using WA in the second plates, we used PDA (Difco^{TM}, BD, France) supplemented with streptomycin antibiotic for the SSI process.

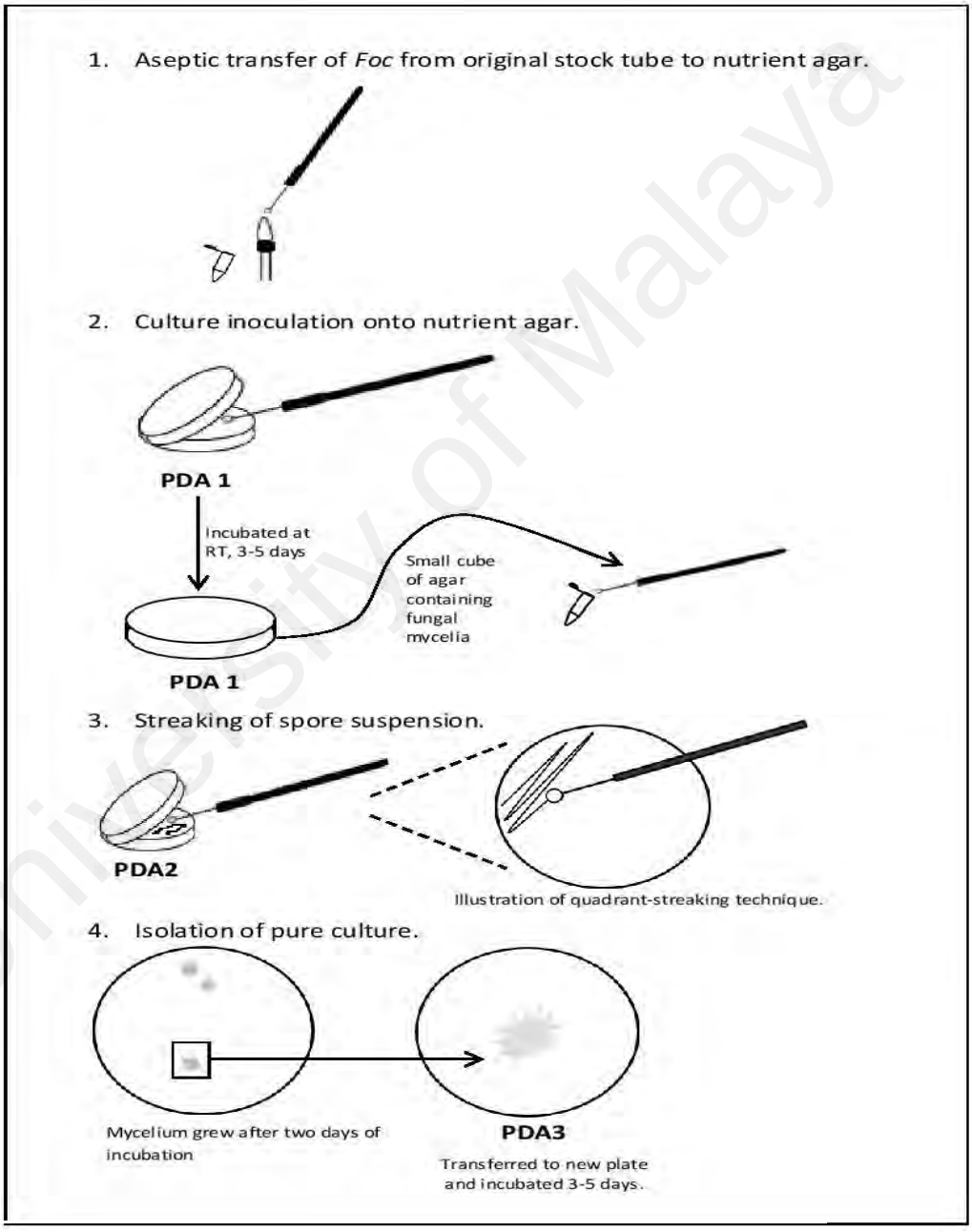


Figure 3.2: Schematic diagram of Single Spore Isolation (SSI) procedure.

3.2.4 Cultural and morphological identification

Each isolate of *F. oxysporum* was identified according to their cultural and morphological characteristics as described by Nelson et al. (1983). The single spore cultures were grown on PDA (Difco^{TM}, BD, France) medium supplemented with streptomycin anatibiotic (Sigma-Aldrich, U.S.A) to determine their growth rate and colony pigmentation. The cultures were incubated at room temperature (27±2℃) for 7 to 10 days in the dark, after which the color of colony was recorded.

3.2.5 Fusarium wilt sampling from infected plants of farmer's field in Jeli, Kelantan

Samples were collected from plants which were showing external wilting symptoms of their elder leaves. The yellowing of the leaves progresses from elder to younger leaves. The leaves collapse slowly, bending at the petiole, near to the midrib, hanging down. This formed a "skirt" of death leaves around the pseudostem.

Those plants showing the above symptoms, sample will be taken from the pseudostem, about 20-30 cm from rhizome. Each sample was taken in triplicate. Once cut, the interior of the affected pseudostem was evaluated. Criteria of an infected sample should have reddish, blackish, or brownish appearance of the vascular lines. The sample is then taken from the center part of the pseudostem. The pseudostem is cut into 15 cm x 15 cm square and placed separately inside sterile plastic bags and labelled accordingly.

Samples were also taken from the rhizome segment (triplicate) of the same infected plant. About 30 cm from the soil surface, the rhizome were taken out and cut into 15 cm x 15 cm squares and placed separately inside sterile plastic bags and labelled accordingly.

Each sample was labelled with:

1. Sample number (one sample per plant)
2. Date
3. Cultivar name of host plant
4. Name of garden, commercial plantation, city
5. Location details

3.2.5.1 Colored vascular strands dissection of the infected sample

Colored vascular strands were dissected immediately after they were collected from the field. Sterile filter paper was used to absorb moisture and an aseptic technique for disease vessels dissection from the sample was applied. This was important to keep any infectious sample away from sterile surfaces. This was to avoid contamination by other co-existing microorganism.

Individual samples were submerged into a 70% (v/v) ethanol (Sigma-Aldrich, U.S.A) solution. The samples were then transferred onto separated filter papers fitted into sterile petri dishes to dry up any moisture under natural conditions. A scalpel blade and forceps, sterilized by flame then submerged in 70% (v/v) ethanol (Sigma-Aldrich, U.S.A) solution, were used to cut the samples into smaller pieces. This step was performed under a laminar flow (Gelman Science) to ensure sterility. The samples continue to be left under the hood for a few more days until the samples are visually dry. Prior use, UV light was switched on in the laminar flow for 15 min before the procedure start, all containers wiped with 70% (v/v) ethanol (Sigma-Aldrich, U.S.A) once placed in the laminar flow.

3.2.5.2 *Fusarium* isolation from infected plant sample

The isolation procedure was performed on the strands as soon as they were dry. Small sections of the vascular vessel tissue were plated onto petri plates using quarter strength PDA (Difco^{TM}, BD, France) supplemented with streptomycin (Sigma-Aldrich, U.S.A). Quarter strength of PDA (Difco^{TM}, BD, France) was used as it is better and faster for fungi sporulation compared to high nutrient conditions. A part from that, the use of quarter strength of PDA (Difco^{TM}, BD, France) able to produce consistent size and shape for fungal identification and characterization (Leslie & Summerell, 2006). Concentration of the antibacterial was adjusted to suit any visually detected bacterial contamination. By performing SSI method using quarter strength of PDA (Difco^{TM}, BD, France) for the whole procedure, a pure culture was harvested.

3.3 General molecular techniques

3.3.1 Genomic DNA (gDNA) isolation of fungal isolates

Genomic DNA extraction methods of fungal isolates were modified from Lin et al. (2009). Prior to extraction, the culture were grown in a Potato Dextrose Broth (PDB) (Difco^{TM}, BD, France) at 25°C in the dark for approximately 1 to 2 week. Grown mycelia were harvested using sterile forceps and blotted-dry on filter paper to remove excessive broth before wrapped in aluminium foil with appropriate labelled. The mycelia was then frozen dry in liquid nitrogen. Frozen mycelia were grounded using a sterile pre-chilled mortar and pestle with the aid of liquid nitrogen. The fine powder from the grounded mycelia (~0.2 – 0.4 g) was immediately transferred into 1.5 ml chilled microcentrifuge tubes. A total of one ml of fungal DNA extraction buffer (Appendix A1i) (instead of 5 ml as been used by Lin et al. 2009) was added to the tube and mixed by vortexed. The samples were heat shocked by placing the tubes into a 65°C water bath with modification

of 15 min. The tubes were then centrifuged to 21, 952 x g by using 70 mm rotor at 4°C for 5 min. The procedure were modified by transferred the supernatant into a new sterile 1.5 ml microcentrifuge tube and 20 µl of RNase A (20mg/ml) (Thermo Scientific, U.S.A) was added. The mixture was mixed well by inverted the tube several times and incubated at 37℃ for three hours followed by transferring into a new sterile 2 ml microcentrifuge tube. An equal amount of phenol:chloroform:isoamyl alcohol (PCIA) (Thermo Fisher Scientific, U.S.A) was added and the mixture was inverted several times to mix well. The mixture was later subjected to incubation at 65℃ for 5 min, cooled down to room temperature before centrifuge at 4℃, 21, 952 x g for 5 min. The aqueous layer was carefully transferred to a new sterile 2 ml microcentrifuge tube without disturbing the organic phase located at the bottom part of the tube. Equal amount of chilled absolute ethanol (Sigma-Aldrich, U.S.A) was added and the mixture was incubated at -20℃ for overnight. The mixture was then centrifuged at 4℃, 21, 952 x g for 10 minutes and the supernatant was discarded. The pellet was washed with 1 ml chilled 75% (v/v) ethanol (Sigma-Aldrich, U.S.A) followed by centrifugation at 4℃, 21, 952 x g for 10 min. The washing step was repeated twice. The pellet was dried using vacuum centrifuge and re-suspended in 30-50 µl of sterile distilled water. The DNA was then stored under -20°C until further used.

3.3.2 Total RNA isolation from plant tissues

A maximum of 1 mg plant root tissues were grounded with a sterile pre-chilled mortar and pestle into fine powder using liquid nitrogen. For this study, the total RNA was extracted using Rneasy® Plant Mini Kits, (Qiagen, Germany) as manufacturer's instructions.

Briefly, a total of 450 µl of Buffer RLT (1% β-mercaptoethanol added as instructed in the protocol) was added to the frozen tissues and the tube was vortexed vigorously. It was then followed by applying a lysate directly into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at 8000 x g. The supernatant was then transferred to a new microcentrifuge tube without disturbing the cell-debris pellet at the bottom of the tube and half volume of 75% (v/v) ethanol (Sigma-Aldrich, U.S.A) was added to the clear lysate, it was then mixed by pipetting. The sample was then applied to an RNeasy mini column placed in a 2 ml collection tube which then subjected to centrifugation at 15,000 x g for 1 min. The flow-through was discarded and the column was inserted into the collection tube. A total of 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 1 min at 8000 x g. The flow-through was then discarded before 500 µl of Buffer RPE was added to the RNeasy column and again centrifuged for 2 min at 10000 x g. For elution, the RNeasy column was transferred to a new 1.5 ml collection tube. Then, 30 µl of RNase-free water was added directly to the column. The column was incubated for 1 min at room temperature, and centrifuged at 10,000 x g for 1 min. The column was discarded and the eluted RNA was located inside the microcentrifuge tube followed by DNase treatment before kept at -80°C until further used.

3.3.3 Agarose gel electrophoresis

Different agarose gel's concentration was used to analyze different sizes of nucleic acid (Table 3.2).

Table 3.2: Concentration of agarose gel used for analyze the integrity of different size of nucleic acid.

Types of nucleic acid	Product size, bp	Concentration of agarose gel, % (w/v)
DNA	< 500 – 700	1.5
DNA/RNA	700 – 1, 500	1.0
Genomic DNA	> 1, 500	0.8

Agarose powder was weighed and placed inside an Erlenmeyer flask. Suitable volume of 0.5 M of 1X Tris borate EDTA (TBE) buffer was then added into the flask. The mixture was then melted by heating in a microwave. At 30 sec time intervals, the flask was removed from the microwave, and the content were swirled to mix well. The procedure were repeat for several times until all agarose has completely dissolved. The mixture needed to be cooled a bit first, before 1 µl of GelStain (TransGen Biotech) was added. GelStain was added in agarose gel solution to ensure the intercalation of DNA with GelStain while viewed using UV light for documentation process. A gel tray was placed into a gel casting apparatus. A gel comb was placed into the gel mold to create the wells. The molten agarose mixture was then poured into the gel mold. Agarose gel was then allowed to solidify. The comb was then removed and place inside gel tank prior used.

A total of 2 µl of 6 X DNA loading dye (Thermo Scientific, U.S.A) and 3 µl of nucleic acid was mixed. The mixtures were loaded onto solid agarose gel in 0.5 M of 1X Tris borate EDTA (TBE) placed inside a gel tank. A total of 4 µl of DNA/RNA size marker

(Thermo Scientific, U.S.A) was loaded along with experimental samples. Enough running buffer was added to cover the surface of the gel. The leads of the gel tank was then attached to the power supply. The gel was electrophoresed at a constant voltage of 100 V for 35 min. The gel was then visualized and photographed under ultraviolet (UV) light using an ultraviolet transilluminator (Bio-Rad ChemiDoc^{TM} MP System).

3.3.4 Determination of nucleic acid integrity by gel electrophoresis

Nucleic acid integrity (clear band) was check by performing gel electrophoresis. Extracted DNA/RNA was visualized using gel electrophoreses technique as mentioned in section Gel electrophoresis (3.3.3).

3.3.5 Determination of DNA concentration and purity

The DNA concentration and purity were determined by measuring optical density (OD) at 260 nm and 280 nm using a Nanodrop Spectrophotometer (Thermo Scientific Nanodrop 2000c). DNA purity was determined by calculating the ratio OD_{260}/ OD_{280}. Sample with purity readings within the range 1.8 to 2.0 were considered pure DNA and selected for the PCR analyses. Every reading were repeated for three times and average was taken and recorded.

3.3.6 Determination of RNA concentration and purity

The RNA concentration and purity were then determined by measuring optical density (OD) at 260 nm and 280 nm using a Nanodrop Spectrophotometer (Thermo Scientific Nanodrop 2000c). Individual samples were measured in triplicate and the average was

taken. OD_{260} of 1.0 is approximately equivalent to 40 µg/ml single-stranded RNA (Lorenz, 2012). Sample with purity readings ~2.0 were selected as pure RNA (Instruction manual, Qiagen, Germany) and will be used for PCR analyses.

3.3.7 Vegetative compatibility analysis (VCG)

All samples were characterized for VCG by Department of Plant Pathology, Stellenbosch University, Western Cape, South Africa. Details method were explain in appendices section A4.

3.4 Molecular detection of *Fusarium oxysporum* f.sp. *cubense*

3.4.1 Species specific detection using Polymerase Chain Reaction (PCR)

Extracted gDNA (>50 ng) with good purity was used as a template for the PCR reaction. All positive isolates of *Fusarium oxysporum* were characterized based on sequence of the ITS region in the rDNA operon of *F. oxysporum* (Mishra et al. 2003). Details of primer pairs used were shown in Table 3.3.

Table 3.3: List of primer used for species detection of *Fo*. Primer pair was synthesized by Integrated DNA Technology (IDT), Singapore.

Target	Primer Name	Sequence (5' – 3')	Expected amplicon size (bp)	Reference
Fusarium oxysporum	Fo-F	ACATACCACTTG TTGCCTCG	340	Das et al., (2012)
	Fo-R	CGCCAATCAATT TGAGGAACG		

The PCR was carried out in an Eppendorf master cycler gradient (Hamburg, Germany, Mastercylcer pro 384) with reaction volume of 25 µl. Target genes were amplified using GoTaq® Flexi Buffer (Promega, U.S.A). An amplification mixture was added to the each individual reaction. The mixture consisted of the template DNA (0.2 µl), $MgCl_2$ (25 µM), 5x PCR buffer (5 µl), dNTP mix (10 µM; 0.4 µl), Taq polymerase (0.125 µl) and primer pairs specific to the targeted gene of ITS region (concentration of 5 µM). The PCR cycling condition was subjected with an initial nucleic acid denaturation at 94℃ for 4 min, followed by 30 cycles of 94℃ for 1 min, 58℃ for 30 sec and 72℃ for 1 min with a final extension of 72℃ for 10 min.

3.4.2 Race specific PCR assay

Determination of race for *Fusarium* isolates was carried out using molecular methods of Polymerase Chain Reaction (PCR). Details of primer sets used was described in Table 3.4 above. The PCR was carried out in an Eppendorf master cycler gradient (Hamburg, Germany, Mastercycler pro 384) with reaction volume of 25 µl. Target genes were amplified using GoTaq® Flexi Buffer (Promega, U.S.A). An amplification mixture was added to the each individual reaction. The components used for PCR was described in Table 3.5 and 3.7 below.

For Race 1 detection, target genes were amplified using gradient PCR with specific primer sets as stated in Table 3.4. This primer set was design by our group based on *Foc* biotype race 1 FPD1 (FPD1) gene, complete coding region (cds) with accession number of EU334870.1 from the GenBank database, The National Center for Biotechnology Information (NCBI). Component mixture as described in Table 3.5 was prepared and 53℃ was selected as the most suitable annealing temperature with PCR cycling conditions as stated in Table 3.6 below.

Race 4 detection of *Fusarium* isolates was performed using *Foc* TR4 VCG01213/012116 primer set. According to Li et al. 2013, this set of primer was designed based on the region that shows nucleotide polymorphisms. They also proved that, this primer set is specific to diagnose *Foc* TR4 (VCG01213/-1216). Results of their study shows that only *Foc* TR4 isolates with VCG01213/01216 generated 452-bp amplicon whereas no amplification was detected in race 1, STR4 and other formae speciales of *Fo*. PCR cycling condition used was described in Table 3.7 below.

Table 3.4: List of primer used for race detection of Foc. All primer pairs were synthesized by Integrated DNA Technology (IDT), Singapore.

Target	Primer Name	Sequence (5' – 3')	Expected amplicon size (bp)	Reference
Foc TR4 VCG01213/ 012116	*Foc*-TR4-F	TGCCGAGAACCA CTGACAA	452	(Li et al., 2013)
	Foc-TR4-R	GCCGATGTCTTC GTCAGGTA		
Foc Race 1	*Foc*-Race1-F	AATCATGTTGCC AACGACAA	245	-
	Foc-Race1-R	GCTCCTCGACAT CACCATTT		

Table 3.5: Components of PCR mix for amplification of *Foc*R1 and *Foc*R4 and fragment using GoTaq® DNA polymerase (Promega, U.S.A.).

No	Components	Final concentration	Volume (1x)
1	Green GoTaq® Flexi buffer (Promega, U.S.A.) (5X)	1 X	5 µl
2	dNTP mix (10 mM)	0.16 mM	0.40 µl
3	$MgCl_2$ (25 mM)	2.50 mM	2.50 µl
4	Forward primer (10 µM)	0.5 µM	2 µl
5	Reverse primer (10 µM)	0.5 µM	2 µl
6	GoTaq® DNA polymerase (5 U/ µl)	1.25 U	0.125 µl
7	Nuclease-free dH_2O	-	Up to 25 µl
8	Template DNA	Variable	50 – 100 ng

Table 3.6: Thermal cycling conditions using GoTaq® DNA polymerase (Promega, U.S.A.) for Race 1 detection.

Condition	Temperature, (°C)	Time	Number of cycle
Initial denaturation	94	4 min	1
Final denaturation	94	1 min	35
Annealing	47 – 57	1 min	
Initial extension	72	1 min	
Final extension	72	10 min	1
Final hold	4	∞	1

Table 3.7: Thermal cycling conditions using GoTaq® DNA polymerase (Promega, U.S.A.) for Race 4 detection.

Condition	Temperature, (°C)	Time	Number of cycle
Initial denaturation	94	4 min	1
Final denaturation	94	1 min	30
Annealing	58	30 sec	
Initial extension	72	1 min	
Final extension	72	10 min	1
Final hold	4	∞	1

3.4.3 Analysis of PCR products

A total volume of 4 µl of PCR product was loaded onto 1% (w/v) agarose gel containing GelStain (TransGen Biotech) alongside with 4 µl of GeneRuler 1 kb or 100 bp DNA ladder (Thermo Fisher Scientific, U.S.A). The gel was electrophoresed as describe in section 3.3.3. PCR products were then purified and stored at -20°C prior to further analysis.

3.4.4 PCR product purification

PCR products were purified from contamination by column purification of the gel slice. These slices were purified to excised desired fragment using Qiaquick Gel Extraction kit (Qiagen, USA) described as follows:

Briefly, the DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The gel slice was weighed in microcentrifuge tube. About 3 volumes of buffer

QG was added to 1 volume of gel. The tube was then incubated in a water bath at 50°C for 10 min. The tube was vortexed for every 2 min during this incubation step. After the gel slice has dissolved completely, the mixture was checked for yellow colour. One volume of isopropanol was added to the sample and mixed by vortexing. A QIAquick spin column was placed in a 2 ml collection tube. The sample mixture was pipetted into the column and centrifuged at 10, 000 x g for 1 min. The flow through was discarded and the column was placed back in the same collection tube. A total of 0.5 ml Buffer QG was added to the column and centrifuged at 10, 000 x g for 1 min. Buffer PE was then added with a volume of 0.75 ml to the column and centrifuged at 10,000 x g for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at 10,000 x g. The column was placed into a clean 1.5 ml microcentrifuge tube. Deionized water (30 μl) was added to the center of the column membrane and allowed to stand for 1 min at room temperature. Finally, the column was centrifuged and the purified DNA was collected in the microcentrifuge tube.

3.4.5 Sequencing analysis

All purified PCR products were sequenced by Integrated DNA Technology (IDT), Singapore. Sequenced results were analyzed using BLAST tool software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) available in National Centre for Biotechnology Information (NCBI) database. Sequence alignment was performed using Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/).

3.4.6 cDNA synthesis

The cDNA was synthesized from total RNA extracted using a *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Beijing TransGen Biotech Co., Ltd) as recommended by the manufacturer. Unique genomic DNA remover was coupled together with *TransScript*® First-Strand cDNA SuperMix to achieve simultaneously removal of DNA and the synthesis of cDNA. Reaction components used in cDNA synthesis were describe in Table 3.8. After thawing, all components of the kit were mixed well by centrifuged and placed on ice. Template mRNA and random primer were added first into a sterile nuclease-free tube and the mixture were incubated at 65℃ for 5 min. It was later chilled on ice for 2 min and other components were added followed by incubation at 25℃ for 10 min, 42℃ for 15 min. The reaction was terminated by heating at 85℃ for 5 sec to inactivate the enzymes. The reverse transcription product was directly used for qRT-PCR applications or stored at -20℃ until further used.

Table 3.8: Reaction components used for cDNA synthesis of total RNA

Component	Volume
Total RNA/mRNA	7 µl
Random Primer (0.1µg/µl)	1 µl
2xTS Reaction Mix	10 µl
TransScript® RT/RI Enzyme Mix	1 µl
gDNA Remover	1 µl

3.4.7 RT-qPCR primer and probe design

The expression profile of two banana defense-related genes was analyzed in cDNA samples obtained from banana roots of highly susceptible *Musa acuminata* cv. 'Berangan' inoculated with *Foc* race 1 (isolate 10201) and race 4 (isolate C1HIR). The *Pathogen related protein 10 (PR-10),* and *Phenylalanine ammonia (PAL)* genes were selected as target gene to assay the expression profile of disease response genes. The *40S Ribosomal protein S2 (RPS2),* and *Glyceraldehyde-3-phosphate (GAPDH)* were reference genes used as a control.

Primer pairs were designed to gene sequences of *Musa acuminata* available in the National Centre for Biotechnology Information (NCBI). Sequences were blast for availability of its coding region of the genes (CDS) and copied by saved in FASTA format. PrimerQuest Tool software was used to design the primer pairs and probe. The optimum parameters were set as melting temperature of 60℃, primer size of 20-24 nucleotide each, a GC content of 45% - 55% and a product size of 100-150 base pairs. The specificity of primer pairs (Figure 3.3 – 3.3.6) selected was confirmed via a BLAST analysis tools in NCBI against *Musa acuminata* sequences. List of primer and probe sequences were listed in Table 3.9. The probe, called PrimeTime probe, was designed specifically labelled at the 5′-end with the fluorescent dye 6-carboxyfluorescein (FAM) as reporter and the 3′-end with ZEN™–Iowa Black® fluorescence quencher (FQ). Location of genes and probes were shown in Figure 3.3 -3.6.

Table 3.9: Primers and probes used for real-time PCR analysis. Primer pairs and probes were synthesized by Integrated DNA Technology (IDT), Singapore. PrimeTime probe, was designed specifically labelled at the 5′-end with the fluorescent dye 6-carboxyfluorescein (FAM) as reporter and the 3′-end with ZEN™–Iowa Black® fluorescence quencher (FQ).

Gene	Accession No	Primer 5'-3'	Primer Tm	Probe 5'-3'	Probe Tm	Amplicon Length
GAPDH	AY821550	For: GTTCCGACTGTTGATGTGTCT Rev: TCCCTCGGATTCCTCCTTAAT	62	AGGCTGCCACCTATGATGAGATCA	67	102
RPS2	LOC103984512	For: GCCATGGTGTCATGGATAGTT Rev: CTCTCTGCTCCTTCCCATTTC	62	TGTTATCCTTCTTCCCGCACTCGC	68	116
PR10	KU942376.1	For: AAAGACCTTCCACTTCACTCC Rev: CTTCGATCGCCTTGTACCTC	62	TTCGTGAAGGACCACGTTGAGGTG	68	107
PAL	EU856392.1	For: TCGATCGGGAACATGTCTTTAG Rev: CTTCTCCTTCTCTCCGTTGTTC	62	AAGTTAAGGCAGGTGCTCGTGGAG	68	119

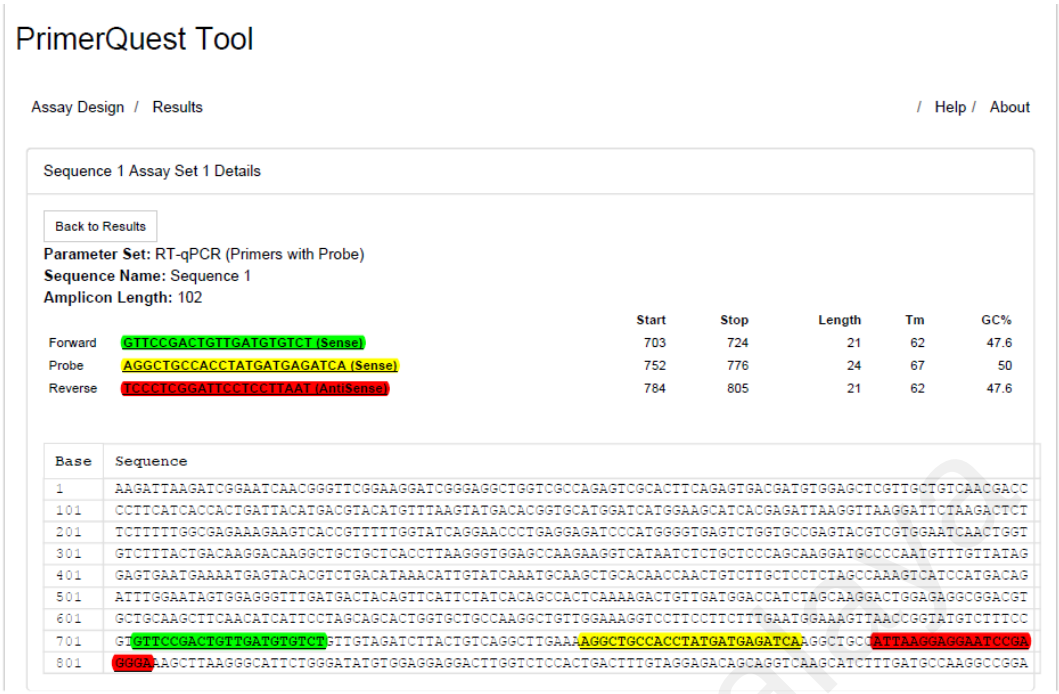


Figure 3.3: Schematic diagram of the position of forward primer, reverse primer and probe for *GAPDH* gene.

Figure 3.4: Schematic diagram of the position of forward, reverse primer and probe for *RPS2* gene.

Figure 3.5: Schematic diagram of the position of forward, reverse primer and probe for *PR10* gene.

Figure 3.6: Schematic diagram of the position of forward, reverse primer and probe for *PAL* gene.

3.4.8 Gene expression analysis using reverse-transcriptase PCR (RT-qPCR)

In order to gain deeper insight into the details of expression patterns exhibited by defense-related genes, 2 steps reverse-transcription PCR (RT-qPCR) experiment was performed for the detection and relative quantification of target genes with defense-response features. qPCR was performed using Applied Biosystem 12K Flexi using Taqman approaches.

Determination of the genes expression stability of the 2 reference genes (*RPS2* and *GADPH* genes) in non-treated and treated root samples was carried out using BestKeeper (Pfaffl et al., 2004).

Reaction mixtures for qPCR of 20µl was describe in Table 3.10 below. All samples were analyzed in triplicate and included triplicate of non-template control reaction for every run. A total of 20 µl of the mixtures were distributed evenly into MicroAMP™ Optical 8-Tube Strips (Applied Biosystem, USA). Amplification cycles were conducted as follows; Initial denaturation at 95 °C for 3 min, thermal cycling was performed for 40 cycles with 95°C for 5 sec, and 60 °C for 30 sec with the fluorescence being read at the end of each cycle.

A standard curve of five serial two-fold dilutions of cDNA was performed to determine the qPCR efficiency of each primer set used. All PCR reactions should displayed a correlation coefficient R^2>0.98 and efficiencies >99%.

Table 3.10: Reaction mixtures for qPCR assay. All components were vortex and quickly centrifuge upon used.

Component	Final concentration	Volume per 20 µl reaction
PrimeTime® Gene Expression Master Mix (2X)	1X	10 µl
Forward & Reverse primers	10 µM	1 µl
Probe	10 µM	0.5 µl
cDNA template	3 pg to 100 ng	2 µl
Nuclease-Free Water	-	5.5 µl

3.4.9 Data analysis

Expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). The most stable reference genes were used as a reference for normalizing the expression data to measure the response of predicted pathogen responsive genes in highly susceptible *Musa acuminata* cv. 'Berangan'.

3.5 Pathogenicity studies

3.5.1 Plant material

Disease-free tissue culture-derived banana *Musa acuminata* cv. 'Berangan' plantlets were obtained from CEBAR University Malaya, Malaysia. The banana plantlets were maintained in Murashige and Skoog (MS) medium for one month. MS active charcoal (10 g/L) was used for the rooting purpose (Gübbük & Pekmezcü, 2004). Plantlets were then transferred to a greenhouse for the acclimatization stage for two months. After two

months of acclimatization, healthy plantlets aging 2 months old with at least 3–5 green leaves, a minimum length of 5 cm of white roots, and stem diameters range from 0.5 to 1.0 cm were chosen for these pathogenicity studies (Figure 3.7).

Figure 3.7: Two month old plantlet of *Musa acuminata* cv 'Berangan' used for pathogenicity studies (bar=2cm).

3.5.2 Acclimatization of banana plantlets

Prior to the acclimatize process of the plantlets, the caps of the tissue culture jars were removed for a period of several days. Plantlets were deflasked from the tissue culture jar and their roots washed free of any growth medium with tap water. They were potted into 100 mm diameter plastic pots containing 3:1 sterilized soil and sand respectively. Acclimatization was carried out in the greenhouse at a controlled temperature of 27℃ ± 2℃ and under plant growth light conditions with a photoperiod of 16 hours. Buah et al. (2000) found 16 hours photoperiod suitable for faster and high yield of banana plantlets.

The plantlet roots were covered with 1 to 2 cm of potting soil and sand mixture up to the upper part of the roots. The plantlets were watered immediately after planting using Hoagland nutrient solution (Sigma-Aldrich, U.S.A). The nutrient solution was watered onto the plantlets for few days in the first week. Followed by weekly interval after the first week and maintained for another 7 weeks. Additionally, half tea-spoon (1.34 g) of nitrogen:phosphorus:potassium (NPK) (15:15:15) fertilizers was added into each pot once every fortnight after 1 month of planting. Hoagland's solution was used during the first month instead of NPK fertilizer to prevent any risk of nutrient deficiency. A part from that, Hoagland's solution is suitable for plant cell culture (Husson et al., 2018). At this stage and under normal growth conditions, all plants reached an approximate height of 15 cm and were ready for *Fusarium* inoculation.

3.5.3 Inoculation Media Optimization

3.5.3.1 Spore suspension cultures

Foc isolate C1HIR was selected from the fungal collection for optimization of the inoculation media. Pure cultures were produced by the single spore isolation routine through a standard streak plate procedure (section 3.2.3). A small chunk of *Foc* isolate (~2 cm x 2 cm) from pure culture grown through SSI procedure was cut and placed inside inoculation media. A total of two different inoculum culture, Potato Dextrose Broth (PDB) (Difco^{TM}, BD, France) and and Armstrong's Liquid Media (ALM) was used. Optimization was performed for both types of inoculation media to choose which one is better suited for *Foc* growth for further experiment. Both inoculum culture media were incubated at room temperature (25-27°C). Both sets of the inoculum culture media were duplicated to test the efficiency of shaken method (continuous shaking on rotary shaker

and manually shaking) towards the media. One set was placed on a rotary shaker at 60 rpm while the other set was shaken twice daily to ensure even distribution of fungus.

Experiment were then repeated with another *Foc* isolates (9888 and 2296) to verify and to see the reproducibility of the results.

3.5.3.2 Spore count

Prior to quantifying the spore concentration, the contents were mixed vigorously and filtered through sterile cheesecloth to separate the mycelium from the spores. The inoculated spore concentration in each inoculation media were counted on 5^{th}, 7^{th} and 9^{th} day of post-inoculation using a hemacytometer. Spores concentration were calculated using formula 3.1 below (Munusamy et al., 2019). The concentration was then adjusted between 1 x 10^5 to 1 x 10^6 spores/ml using sterile distilled water. Suitable inoculation media were then chosen to participate in the experiment of pathogenicity studies based on the data obtained in this section.

$$Spore\ concentration = \frac{Total\ of\ spores\ in\ all\ squares\ counted}{No.\ of\ squares\ counted}\ x\ 2\ (dilution\ factor)\ x\ 1000$$

Formula 3.1: Calculation for spore concentration.

3.5.3.3 Statistical analysis

The data generated from spores concentration were subjected to statistical analysis of one way ANOVA and Tukey HSD Multiple Comparison Test. Statistical significance of all terms was asserted at the 95% confidence interval ($p<0.05$). The analyses were undertaken using SPSS 16.0 software (SPSS Inc., U.S.A).

3.5.3.4 Viability test

Viability test was performed on the 5th day of post-inoculation by spreading 50 to 100 µl of a spore suspension onto a fresh PDA plate. The PDA plate was supplemented with streptomycin antibiotic and placed under the dark for 36 to 48 hours with the cap flipped upside down. The number of recovery colonies can be illustrious from the all-or-nothing states of life and death by the use of a quantifiable index between 0 and 1 (or 0% and 100%).

3.5.4 Inoculum preparation

A liquid medium, PDB (Difco™, BD, France) was chose to prepare the inoculum for pathogenicity studies. PDB (Difco™, BD, France) medium was concocted in 1L schott bottle and autoclaved at 121℃ with 15 psi for 20 min. After the medium was naturally cooled down, it was inoculated with *Foc* isolate. Five PDA (Difco™, BD, France) agar stabs of 1 mm^3 were taken from a uniformly growing fungal culture on PDA (Difco™, BD, France) onto the prepared PDB (Difco™, BD, France) media using sterile inoculation loop. Inoculum culture media were incubated at room temperature (25-27°C) and shake (up and down for several times) twice daily for 7 days. Viability was confirmed on the 5th day of post-inoculation (section 3.5.3.3). After incubation for 7 days, the

inoculum medium was poured through cheesecloth to separate spores from mycelia. The conidia concentration in the suspension was determined with a hemacytometer (section 3.5.3.2) and adjusted with sterile distilled water to achieved final concentration of 10^5 to 10^6 spores per ml.

3.5.5 Infection of plants and bioassay protocol

A total of 23 biological replicates of acclimatized *Musa acuminata* cv. 'Berangan' plantlets were inoculated with *Foc* C1HIR inoculum for our standardized bioassay experiment with two technical replicates were perform. After gaining useful knowledge and all precautions were taken into consideration, the experiment were then repeated with 4 different treatment of *Foc* inoculum (isolate 2306 and 10201 for race 1 while isolate 2296 and 9888 were used for race 4 *Foc*). The acclimatized plantlet were first carefully uprooted from their 100 mm plastic pots and the roots slightly injured by hand through washing them with water to clean the roots from soil residue. The *Foc* spore suspension was then placed on a big container. For inoculation, the whole cleaned roots were immersed directly onto the inoculum media for two hours. The plantlets were then replanted in the original potting soils with the appropriate labelling. Sterile distilled water was used as 'inoculum' for the negative control plantlets. Later the plants were transferred to a double-container apparatus and maintained in the greenhouse through Randomized Complete Block Design (RCBD) (Figure 3.8).

A double compartment container consisting of a tray measuring 43 × 29 × 9 cm, which fits snugly into another, larger, outer tray measuring 46 × 31 × 20 cm was used to place the infected plants material (Figure 3.9). The plantlets were watered with only tap water every 2 days. Hoagland's solution (Sigma-Aldrich, U.S.A.) was used as fertilizer once a week. The plants were maintained in the growth room at ambient temperature 25 ± 2℃

with a 16 hours photoperiod. The pathogen was re-isolated from representative diseased plants to prove Koch's Postulates.

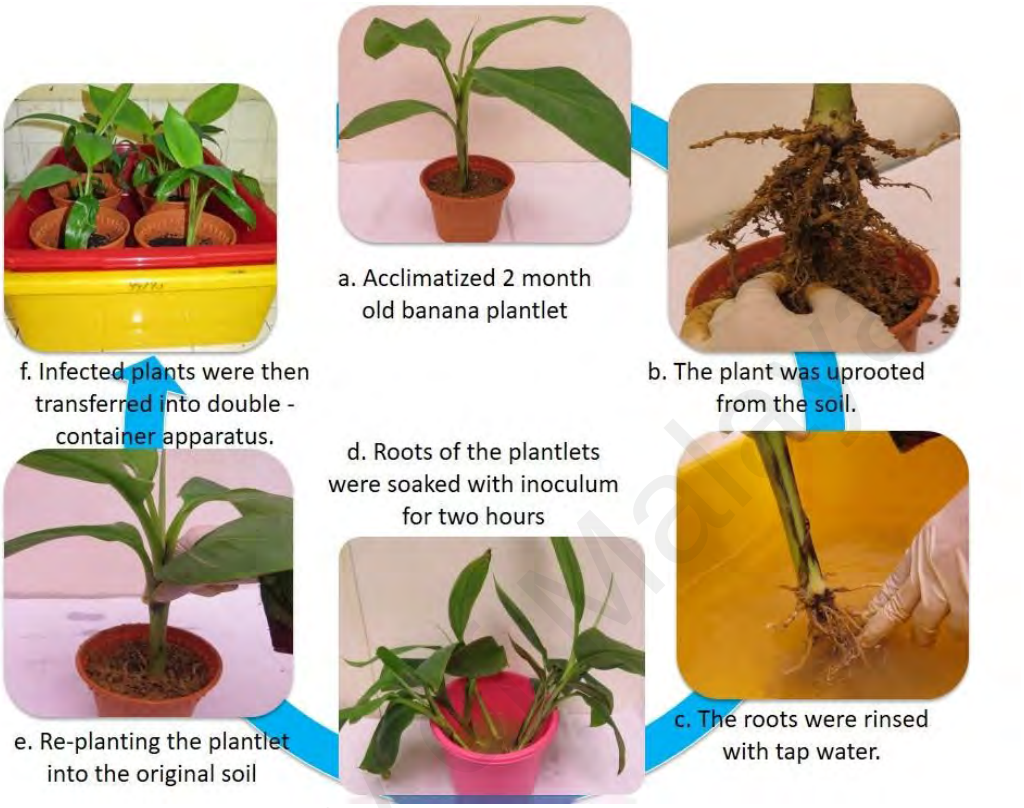


Figure 3.8: Flow chart illustrating the Fusarium wilt bio-assay procedures.

Figure 3.9: The 'Double-tray' method allows contaminated water to be contained in the lower tray that can be easily treated with sodium hypochlorite (1000ppm/L of chlorine) prior to disposal; a) Upper tray with punched holes (4 x 3 =12 holes per tray) (bar = 4 cm); b) 6 plantlets were placed in each tray (bar = 2.5cm).

3.5.6 Sample collection from infected plants for molecular identification

Infected banana roots were washed with tap water in order to remove all adhering soil particles. Roots pieces approximately 50 mm in length were cut, washed using sterile distilled water, damp dried on tissue paper and subjected to deep freezing using liquid nitrogen. Freeze samples were then placed inside appropriate container and stored inside -80℃. Samples were collected on three time points of post-inoculation, 0 days of post-inoculation (dpi), 2 dpi and 4 dpi. For every time point, three plants were sacrifice and label accordingly prior to storage. Samples were subjected to total RNA isolation (section 3.3.2) for molecular analysis of qPCR (section 3.4.6-3.4.8).

3.5.7 Field Emission Scanning Electron Microscope (FESEM) observations

Samples were processed and prepared following the methods of Rahman and co-researchers, (2010) for FESEM observation. The specimens were dehydrated with normal air dry and adhered onto aluminum specimen mounts with carbon tape. The specimen were coated with platinum for 30 sec and photographed using FEG Quanta 450, EDX-OXFORD electron microscope (Germany).

3.5.8 Assessment of disease symptoms and data collection

Visual assessment of the Fusarium wilt external symptoms was made. They were scored with the percentage of infection of each symptom at the end of the 5th week of post-inoculation recorded. A 5-point scale was used for wilting and yellowing where these value determined the leaf symptom index (LSI) of the cultivar (Table 3.11).

For internal symptom, infected plantlets were carefully uprooted from the pots and the pseudostem removed, leaving behind the rhizome and roots region. The plants were washed to remove soil residue from the roots. Their rhizomes were vertically cut into half to observe the brownish/darkish discoloration. Scores for rhizome infection intensity were made and 8-point scale (Table 3.11) was used to compare the level of discoloration to get the rhizome discoloration index (RDI). Both LSI and RDI were then been used to obtain the disease severity index (DSI) that was used to determine susceptibility or resistance level of the cultivar tested.

Table 3.11: Leaf symptom index (LSI) and rhizome discoloration index (RDI) scale value used in the evaluation of plants' response against *Foc* infection.

Leaf symptoms	Score	Leaf symptom
index (LSI)	1	- No streaking or yellowing of leaves.
	2	- Slight streaking and/or yellowing of lower leaves. Plant appears healthy.
	3	- Streaking and/or yellowing of most of the lower leaves.
	4	- Extensive streaking and/or yellowing on most or all of the leaves.
	5	- Dead plant
Rhizome discoloration index (RDI)	1	- No discoloration of tissue of stellar region of rhizome or surrounding tissue.
	2	- No discoloration of stellar region of rhizome; discoloration at junction of root and rhizome.
	3	- Trace to 5% of stellar region discoloured.
	4	- 6-20% of stellar region discoloured.
	5	- 21-50% of stellar region discoloured.
	6	- More than 50% of stellar region discoloured.
	7	- Discoloration of the entire rhizome stele.
	8	- Dead plant

3.5.9 Final disease translation

For pathogenicity test experiment, final disease translation was computed using Disease Severity Index (DSI) using data from the LSI and the RDI according to Mak at al. (2004). The formula for SDI is as shown below in Formula 3.2.

$$\text{DSI} = \frac{\sum(\text{Number on scale x Number of seedlings in that scale})}{\sum(\text{Number of treated seedlings})}$$

Formula 3.2: Formula used to calculate Disease Severity Index (DSI). DSI is calculated based on the LSI and RDI scores value to determine the disease response of infected plants from yellowing of leaves and discoloration of rhizomes, respectively.

The final DSI value is correlated with one of the four designations (Table 3.12). In the case when one of the indexes was designated as susceptible, then the cultivar was regarded as susceptible against that particular isolate. Similarly, if one of the scores (either RDI or LSI) indicated it as tolerant while the other as resistant, then the cultivar was considered as only tolerant. Once both disease indications are resistant, then the cultivar be considered as resistant.

Table 3.12: Interpretation of the Disease Severity Index (DSI) scales.

DSI Scales for LSI	DSI Scales for RDI	Translation
1.0	1.0	Resistant
Between 1.1 and 2	Between 1.1 and 3	Tolerant
Between 2.1 and 3	Between 3.1 and 5	Susceptible
Between 3.1 and 5	Between 5.1 and 8	Highly susceptible

CHAPTER 4: RESULTS

4.1 Morphological Characterization

A total of forty two fungal colonies obtained from Prof Baharuddin formed on PDA (Difco^{TM}, BD, France) medium supplemented with streptomycin were identified as *Fusarium* spp. based on the morphology shown by each colony using the *Fusarium* synoptic keys for species identification of Leslie et al. (2006). These isolates were identified as *F. solani*, *F. oxysporum* and other *Fusarium* spp. The other colonies were identified at the genus level only as *Fusarium*. Details results of identified morphological characteristics are shown in Table 4.1. A total of 31 *F. oxysporum* isolates were selected for further study. Example of culture is shown in Figure 4.1.

Figure 4.1: Typical growth of *Fusarium* isolate.

Table 4.1: Morphological characteristics of 42 fungal isolates on PDA (Difco™, BD, France) culture.

No	Isolates No	Species identification	Colony colour	Pigmentation
1.	235	Other *Fusarium* spp	White	White
2.	236	*F. solani*	White	White
3.	239	Other *Fusarium* spp	White	White
4.	240	*F. solani*	White	White
5.	241	*F. solani*	White	White
6.	243	*F. solani*	White	White
7.	255	Other *Fusarium* spp	White	White
8.	523	*F. oxysporum*	White	White
9.	1454	*F. oxysporum*	Purple	Purple
10.	1462	*F. oxysporum*	White	Purple
11.	2279	*F. oxysporum*	White	Purple
12.	2280	*F. oxysporum*	White	White
13.	2281	*F. oxysporum*	White	Purple
14.	2282	*F. oxysporum*	White	Purple
15.	2284	*F. oxysporum*	White	Purple
16.	2288	*F. oxysporum*	White	White
17.	2290	*F. oxysporum*	White	White
18.	2291	*F. oxysporum*	White	Purple
19.	2293	Other *Fusarium* spp	White	Purple
20.	2294	*F. oxysporum*	Light purple	Purple
21.	2295	*F. oxysporum*	White	White
22.	2296	*F. oxysporum*	White	White
23.	2305	*F. oxysporum*	Purple	Purple
24.	2306	*F. oxysporum*	White	Purple
25.	2307	*F. oxysporum*	White	White
26.	2327	*F. oxysporum*	Purple	Purple
27.	2328	*F. oxysporum*	White	Purple
28.	2330	*F. oxysporum*	White	Purple
29.	2331	*F. oxysporum*	White	White
30.	2332	*F. oxysporum*	White	White
31.	2460	*F. oxysporum*	Light purple	Purple
32.	2461	*F. oxysporum*	Light purple	Purple
33.	2462	*F. oxysporum*	White	White
34.	2468	Other *Fusarium* spp	White	Purple
35.	2469	*F. oxysporum*	White	Purple
36.	2471	Other *Fusarium* spp	White	Purple

Table 4.1, continued.

37.	2472	*F. oxysporum*	Purple	Purple
38.	9886	Other *Fusarium* spp	White	White
39.	9887	*F. oxysporum*	Light purple	Purple
40.	9888	*F. oxysporum*	White	White
41.	9889	*F. oxysporum*	White	Purple
42.	10201	*F. oxysporum*	White	White

4.2 General molecular technique

Extracted genomic DNA was evaluated for its integrity (Figure 4.2) and quality (Table 4.2) prior to PCR identification.

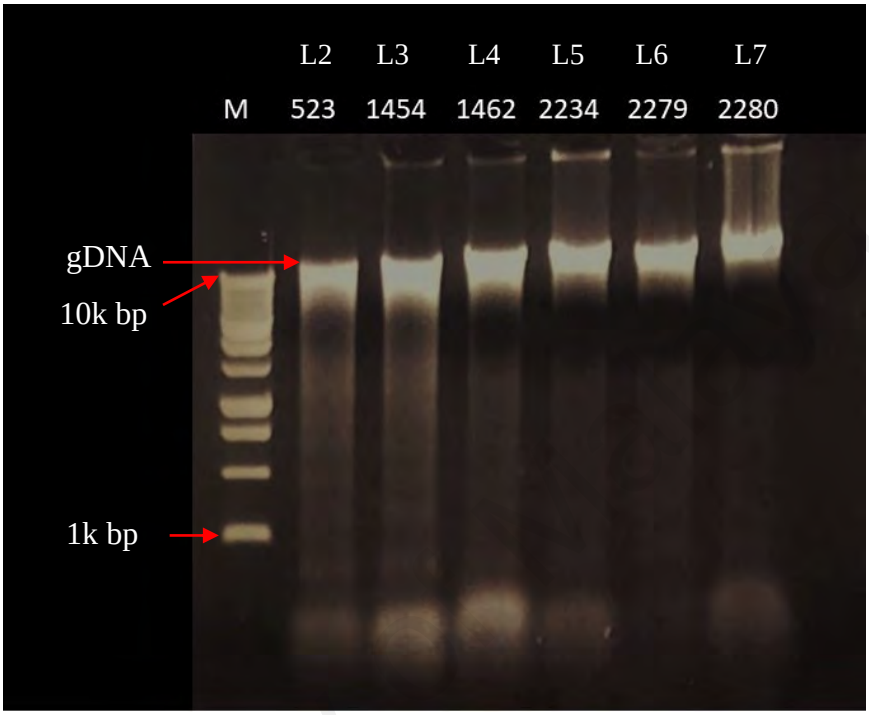


Figure 4.2: Representative of extracted genomic DNA of *Foc*. Lane M: 1 kb DNA ladder (Promega), Lane 2: *Foc* isolate 523, Lane 3: *Foc* isolate 1454, Lane 4: *Foc* isolate 1462, Lane 5: *Foc* isolate 2234, Lane 6: *Foc* isolate 2279, Lane 7: *Foc* isolate 2280.

Table 4.2: DNA concentration and purity of extracted samples of representative *Foc* isolates.

Sample ID	Nucleic Acid Concentration (µg/µL)	A260	A280	260/280	260/230
523	1.1726	28.5	14.9	1.91	0.85
1454	1.2277	26.5	14.1	1.86	0.74
1462	1.0345	22.9	12.05	1.90	0.80
2234	1.7061	32.0	17.02	1.88	0.61
2279	1.7207	24.1	13.03	1.84	0.80
2280	2.0122	31.8	16.06	1.98	0.85

4.3 Molecular characterization

Two different race specific primers, were used to screen against a total of 31 *F. oxysporum* isolates. Race 1 specific primers produced a distinct band with approximate size of 250 base pairs (bp) in length (Figure 4.3). Analysis of race 4 specific primers produced a discrete band with size of base pairs approximately around 500 bp (Figure 4.4). Examinations of the 31 isolates revealed that a total of 7 isolates falls into race 1 group and 17 isolates were grouped into race 4. There were 7 isolates which could not be identified and the details of the race specific identification are summarized in Table 4.3 below. As for VCG, out of 31 isolates sent for identification, 22 isolates gave a positive VCG identification (Table 4.3).

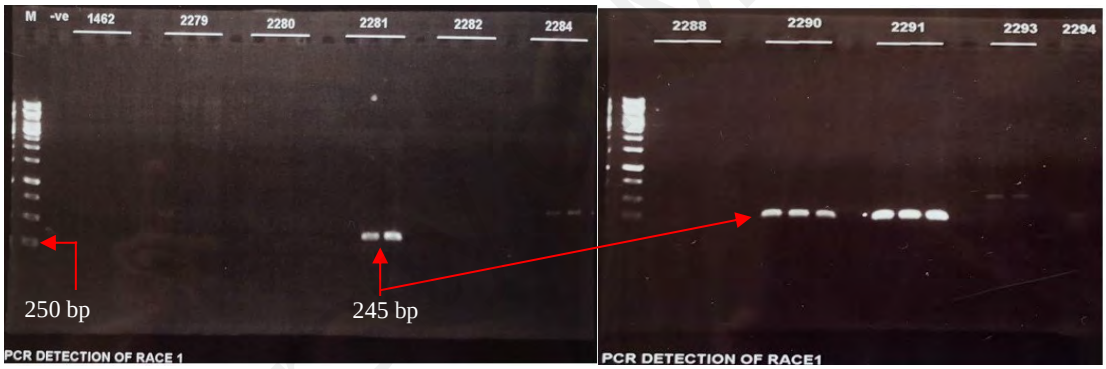


Figure 4.3: Race 1 identification of representative of *Foc* isolates.

Figure 4.4: Race 4 identification of representative of *Foc* isolates.

Table 4.3: Molecular characterization of 31 samples *F. oxysporum.*

No	Isolate code	Species identification	Race	VCG[1]
1.	523	*F. oxysporum*	Race 4	01213/16
2.	1454	*F. oxysporum*	Race 4	01213/16
3.	1462	*F. oxysporum*	Race 4	01213/16
4.	2234	*F. oxysporum*	Race 4	01213/16
5.	2279	*F. oxysporum*	Race 4	01213/16
6.	2280	*F. oxysporum*	-	01217
7.	2281	*F. oxysporum*	Race 1	In process
8.	2282	*F. oxysporum*	Race 4	01213/16
9.	2288	*F. oxysporum*	Race 4	01213/16
10.	2290	*F. oxysporum*	Race 1	1218
11.	2291	*F. oxysporum*	Race 1	1217
12.	2294	*F. oxysporum*	-	1218
13.	2295	*F. oxysporum*	Race 1	In process
14.	2296	*F. oxysporum*	Race 4	01213/16
15.	2305	*F. oxysporum*	Race 4	01213/16
16.	2306	*F. oxysporum*	Race 1	Not compatible to Lineage 6 VCGs
17.	2307	*F. oxysporum*	Race 1	Not compatible to Lineage 6 VCGs
18.	2327	*F. oxysporum*	Race 4	01213/16
19.	2328	*F. oxysporum*	Race 4	01213/16
20.	2330	*F. oxysporum*	Race 4	01213/16
21.	2331	*F. oxysporum*	-	In process
22.	2332	*F. oxysporum*	-	01213/16
23.	2460	*F. oxysporum*	-	In process
24.	2461	*F. oxysporum*	Race 4	01213/16
25.	2462	*F. oxysporum*	Race 4	01213/16
26.	2469	*F. oxysporum*	Race 4	01213/16
27.	2472	*F. oxysporum*	-	In process
28.	9887	*F. oxysporum*	-	In process
29.	9888	*F. oxysporum*	Race 4	01213/16
30.	10201	*F. oxysporum*	Race 1	In process
31.	C1H1R	*F. oxysporum*	Race 4	01213/16

[1] VCG is still in process up to 20/9/19.

4.4 Field sampling of vascular tissue colonization

An external observation is made of a candidate plant in the farmer's field in Jeli, Kelantan. To be qualified for collection, the plant must exhibit Fusarium wilt symptoms (wilting and yellowing of the older leaves, starting around the margins and progress towards the midrib area as shown in the Figure 4.5). Focusing on the area as denoted by the red arrows in Figure 4.5 (A), it is observed that the midrib area of the leaf is still green in colour, but leaf margin area is yellowing (indicating the wilting symptom). More definitive symptoms are found by cutting the pseudostem of the plant (figure 4.5 (B)), where the typical brown discoloration was noted on dissection. Samples were then collected and placed inside sterile plastic bags and brought back to our facility for further analysis and diagnosis.

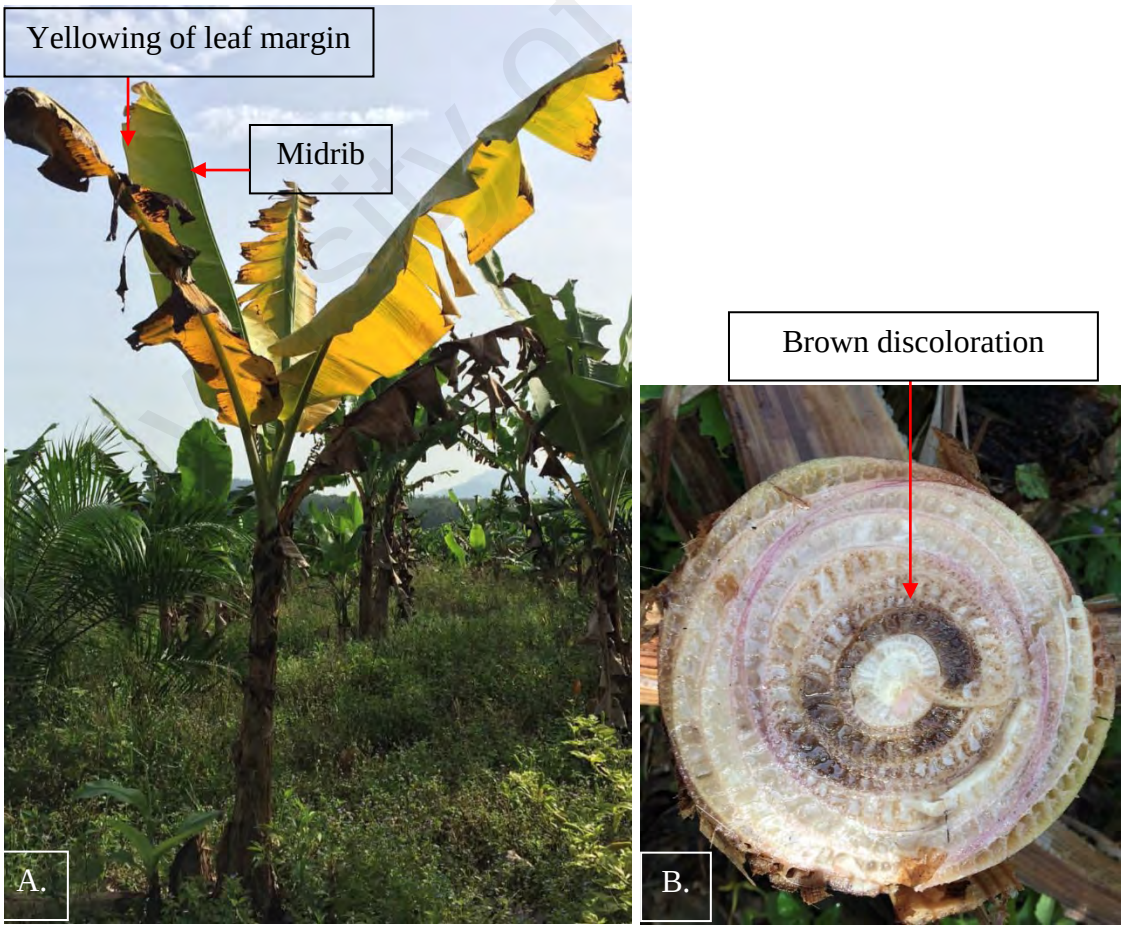


Figure 4.5: Fusarium wilt symptoms observed in farmer's field in Jeli, Kelantan. A: External wilting symptom observed on the banana plant. B: Brown discoloration of pseudostem on the same banana plant.

A total of 60 colored vascular strands were collected from rhizome and pseudostem of infected banana plant. Each of the 60 vascular strands were placed on a sterile filter paper in the Petri plates and placed under laminar flow for 35 hour as shown in figure 4.6. The dried colored strands were then placed on quarter PDA (Difco™, BD, France) supplemented with streptomycin antibiotic (1.2 ml /240 ml of media). The plates was labeled as 'PDA1' and incubated in the dark at 25℃ for 7 days.

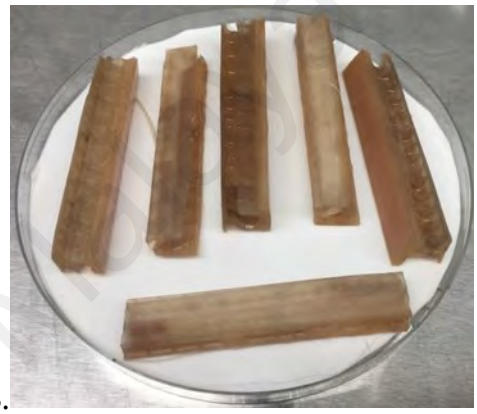
A.  B. 

Figure 4.6: Infected coloured vascular strands from (A) rhizome and (B) pseudostem of infected banana plant respectively. Samples were placed on sterile filter paper inside sterile Petri plates. Samples were then dried for 35 hour in the laminar flow until visually dry.

After 7 days of incubation, 12 of the 60 plates produced fungal colonies exhibiting *Fusarium* morphology. The other plates were contaminated with a wide range of bacteria (not shown in the figure). All colonies on quarter strength PDA (Difco™, BD, France) was growing slow and dark-violet pigmentation were observed on all plates.

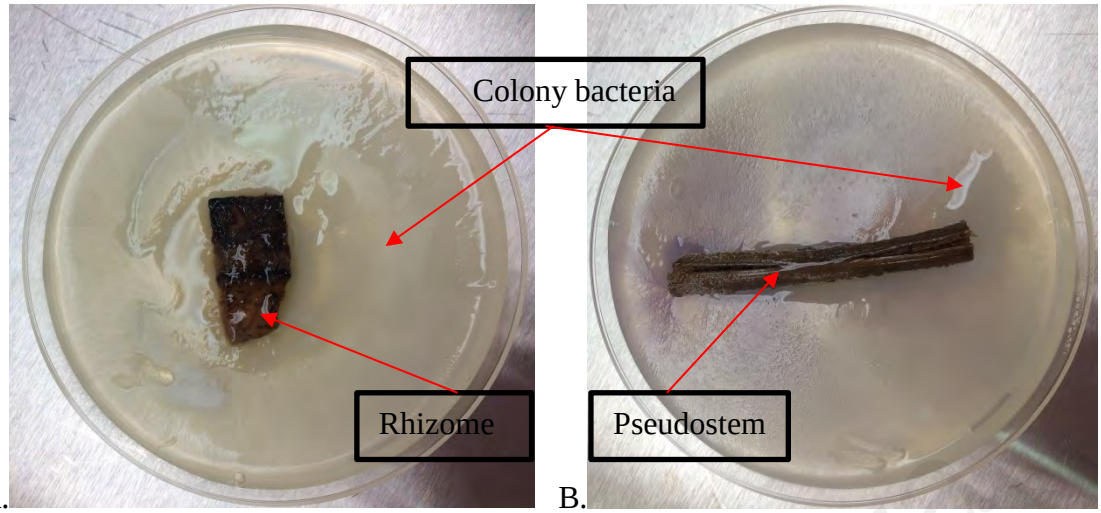
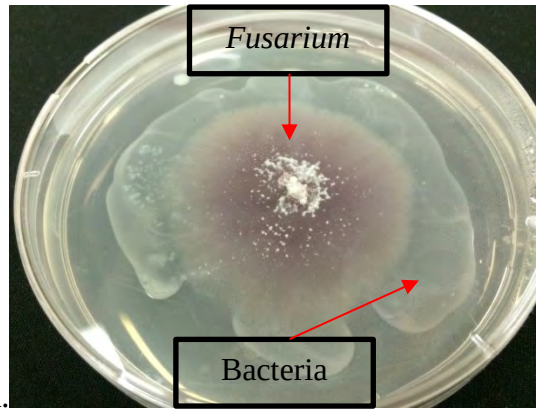


Figure 4.7: Fungal colony growth of *Fusarium oxysporum* from infected coloured vascular strands (A) rhizome and (B) pseudostem of infected banana plant respectively after 7 days of incubation period in dark at 27℃.

The colony growth that could be observed in Figure 4.7 was badly influence by the growth of bacteria. The masking effect by the bacteria caused the fungal growth to be hindered as the colony can only be seen after day 3 of incubation. The fungal colony was observed to appear only after the bacteria had started to grow on the media. The colony of bacteria can be seen on the outside of the fungal colony.

The experiment was then repeated again but the concentration of antibiotic used was doubled in the quarter strength PDA (Difco^{TM}, BD, France). A scrape of the fungal colony was collected from the initial (PDA1) and placed on fresh quarter strength PDA (Difco^{TM}, BD, France) supplemented with of streptomycin (2.4 ml/240 ml of media).This sampled was labeled as PDA2. The same incubation parameters were used and the fungal colony growth was inspected daily. Contaminated plates were discarded immediately. Fungal colony growth on day 7 was inspected. Figure 4.8 shows the result of the fungal growth after the incubation.

A. 

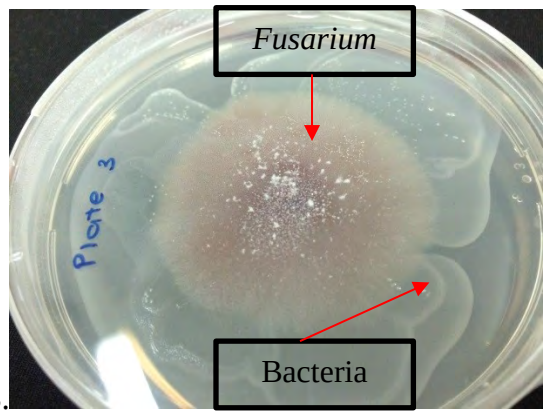
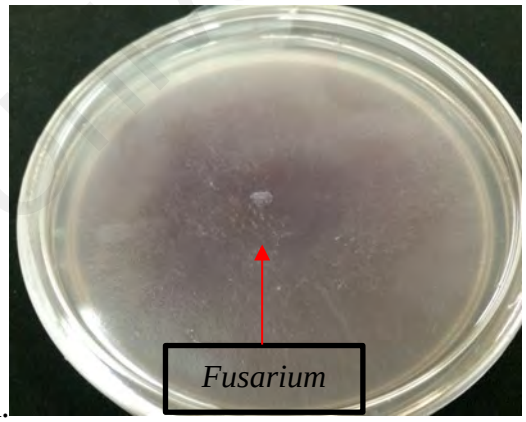
B. 

Figure 4.8: Fungal colony growth after 7 days of incubation period. Colony was the sub-culture taken from PDA1 culture placed in the quarter strength PDA (DifcoTM, BD, France) supplemented with double-up antibiotic concentration.

The second attempt of culturing was still masked by the growth of bacteria as can be seen from the Figure 4.8. The bacteria can be clearly detected surrounding of the fungal colony though the effect was reduced. The experiment was then repeated with the variation of the concentration of streptomycin. For this iteration, the concentration of streptomycin was set to 3.6 ml/240 ml of media. The plates were labelled "PDA3". The same incubation parameters were used. Colony growth on day 7 of incubation can be observed in Figure 4.9 below.

A. 

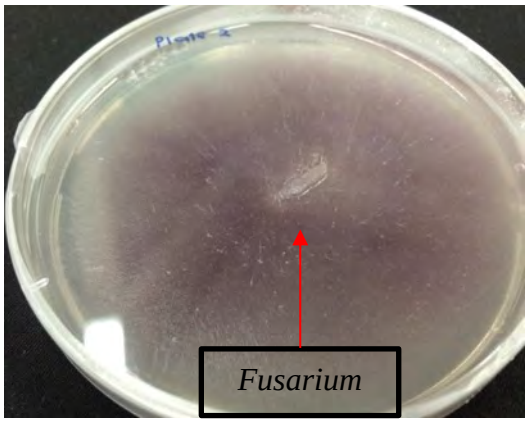
B. 

Figure 4.9: Fungal colony growth on fresh quarter strength PDA (DifcoTM, BD, France) supplemented with 3.6 ml of streptomycin after 7 days of incubation period from sub-culture PDA2.

On the basis of morphology and characteristics of the culture colony, the fungal isolate was identified as *Fusarium*. Clear colonies of fungi were successfully cultured after the third transfer onto new quarter strength PDA (Difco^{TM}, BD, France) supplemented with 3.6 ml of streptomycin antibiotic for each 240 ml of PDA (Difco^{TM}, BD, France).

Fungal colonies from PDA3 were then used for the Single Spore Isolation (SSI) procedure. Pure isolates from a single spore were successfully cultivating after a scrape of sporulating hyphae from PDA3 were collected and dissolved into 2 ml of sterile distilled water in 2 ml eppendorf tubes. Pure isolates were then used for the extraction method prior to the PDB (Difco^{TM}, BD, France) culturing.

The usual SSI methods need to be adjusted to acquire the optimal result. The original protocol suggests smudging a scrape of sporulating hyphae from PDA3 and dissolving it in 1 ml of sterile distilled water. However, the results show that no single colonies were developed. The colonies were growing proximal to each other proving that the sporulating hyphae were high in concentration. The experiment was repeated with varying the concentration of sporulating hyphae. The sporulating hyphae were diluted in 2 ml of sterile distilled water. Now, single colonies were developing. The SSI was adapted for the following steps of the experiment.

4.4.1 DNA quantification and quality of extracted Fusarium sample

The concentration of the extracted fungal DNA obtained from infected banana plant was estimated by using Nanodrop (Thermo Scientific Nanodrop 2000c) and shown in Table 4.4 below.

Table 4.4: DNA concentration and purity of extracted samples of vascular tissue from field sampling.

Sample ID	Nucleic Acid Concentration (ng/µL)	A260	A280	260/280	260/230
Pseudostem 1	1325.2	26.504	14.109	1.87	0.85
Pseudostem 2	544.8	10.896	6.874	1.59	0.74
Rhizome 1	1143.7	22.873	12.299	1.85	0.80
Rhizome 2	550.0	11.001	6.801	1.62	0.61

Genomic DNA quality and purity were also tested by means of electrophoresis in 1% (w/v) agarose gels, followed by staining with gel rad. DNA obtained was an intact band of fine quality as shown in Figure 4.10 below.

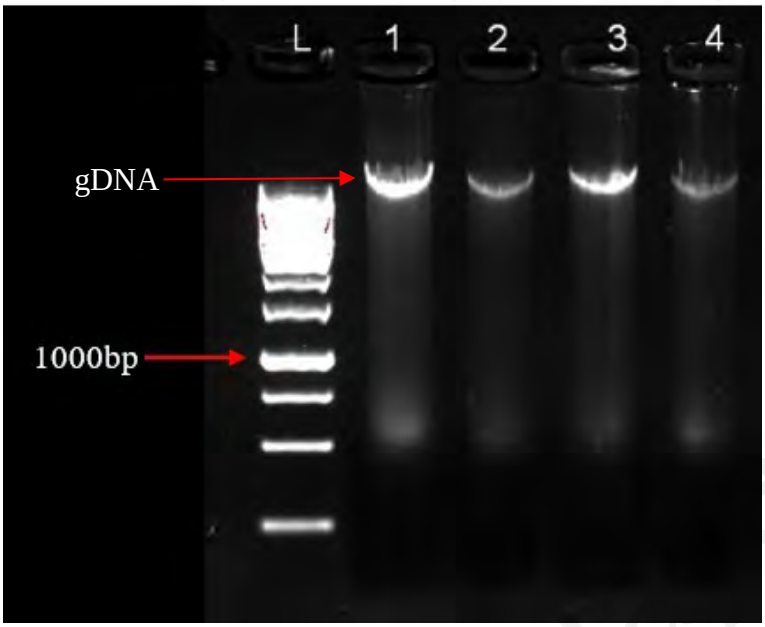


Figure 4.10: Agarose gel electrophoresis of genomic DNA isolated from infected colored vascular strands of rhizome and pseudostem of infected banana plant. Lane L corresponds to 1kb DNA ladder, Lanes 1 & 2, fungal isolates from pseudostem, and Lanes 3 & 4, fungal isolates from rhizome of infected banana plant.

A 260/280 ratio of DNA range from 1.70 to 2.0 was chosen for further analysis which confirmed that it is free from protein impurities. The amount and quality of genomic DNA obtained by the present protocol were suitable for PCR amplification and other molecular essays. Based on the concentration and purity readings, sample ID Pseudostem 1 and Rhizome 1 were chosen for further analysis.

4.4.2 Molecular analysis

PCR amplification for *Fusarium oxysporum* identification using the primers Fo-F/Fo-R, consistently produced the expected sized PCR amplicon of 340 bp from the plant samples showing characteristic panama disease symptoms banana pseudostem and rhizome collected from infected field at an annealing temperature of 58℃ (Figure 4.11).

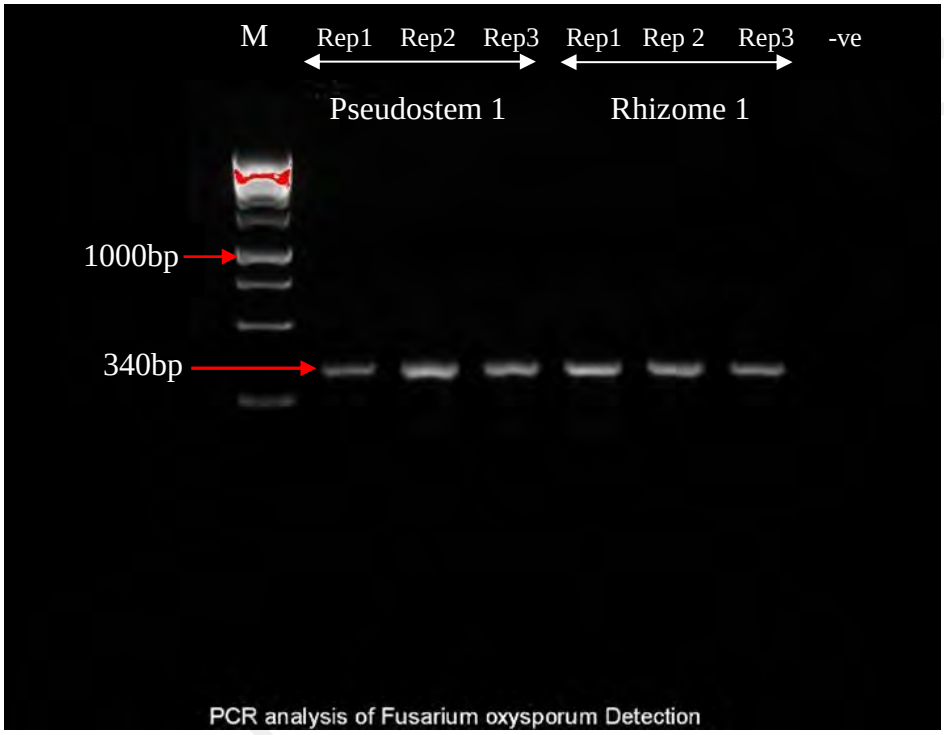


Figure 4.11: PCR amplification of *Fusarium oxysporum* by the primers Fo-F/Fo-R from extracted sample of vascular tissue from field sampling in Jeli, Kelantan. . Lane M: 1 kb DNA ladder (Promega), Lane Rep1, Rep2, and Rep 3 of Pseudostem 1: sample from sample ID Pseudostem 1; Lane Rep1, Rep2, and Rep3 pf Rhizome 1: sample from sample ID Rhizome 1; Lane -ve: water (negative control).

PCR for Race 1 *Foc* detection with the primer pair *Foc*-Race1-F/*Foc*-Race1-R generated an amplicon approximately 245 bp in size in all samples tested (Figure 4.12).

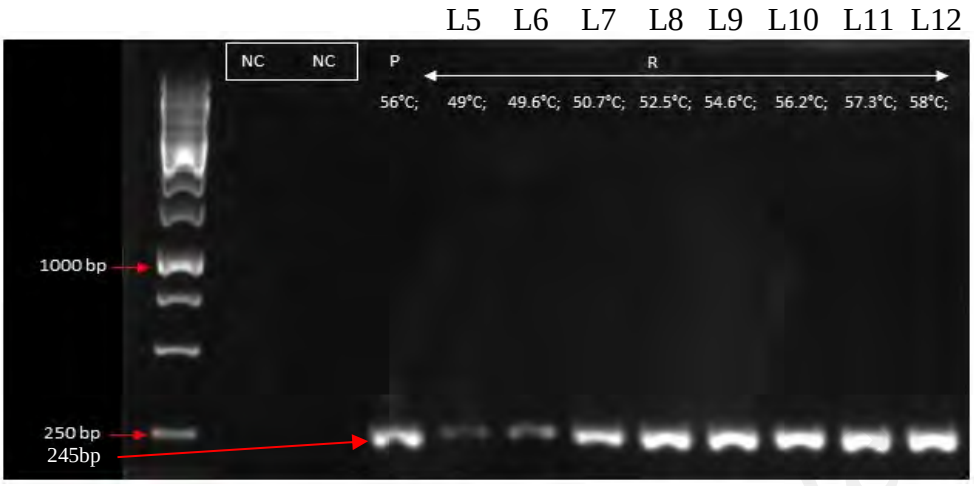


Figure 4.12: PCR amplification of Race 1 detection able to generate amplicon of 245 bp in all samples. Lane 1; 1 kb DNA ladder (Promega), Lane 2 & 3; -ve control Lane 3; Lane 4; Pseudostem sample for 56°C annealing temp; Lane 5-12; Rhizome sample onn 49°C, 49.6°C, 50.7°C, 52.5°C, 54.6°C, 56.2°C, 57.3°C and 58°C annealing temperature respectively.

PCR amplification for *Foc* Race 4 detection using primer pair *Foc*-Race4-F/*Foc*-Race4-R give a negative results as shown in Figure 4.13 below.

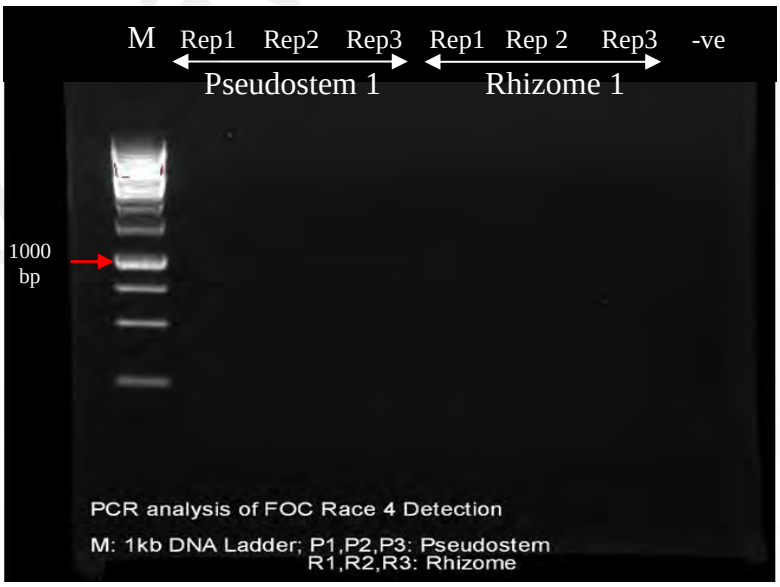


Figure 4.13: PCR amplification of *Foc* TR 4 from extracted sample of vascular tissue from field sampling in Jeli, Kelantan. . Lane M: 1 kb DNA ladder (Promega), Lane Rep1, Rep2, and Rep 3 of Pseudostem 1: sample from sample ID Pseudostem 1; Lane Rep1, Rep2, and Rep3 pf Rhizome 1: sample from sample ID Rhizome 1; Lane -ve: water (negative control).

4.4.3 Sequencing analysis

Raw data of sequencing results were attached in Appendices section D. Sequence from reverse primer was reverse compliment using ClustalW. Reverse compliment results were also attached in Appendices section D. The complete sequence of sample ID of Pseudostem 1 from vascular sample taken from farmer's field in Jeli, Kelantan were then analyzed against the available sequences in the GenBank deposited in the NCBI website. Druzhinina et al., (2005) in their report emphasized that database placed inside GenBank covers many sequences of *Fusarium* isolates which can be considered for identification purpose. Sequence data submitted in GenBank with Accession No. MF491831.1 showed 99% nucleotide identity through BLAST analysis with the amplified sequence by using species specific primer set reported for species detection of *Fo* (Figure 4.14).

Similarly, the BLASTn search analysis of nucleotide sequence on the NCBI website indicated that the PCR amplified product for race detection showed 93% similarity with the sequence of *Fusarium oxysporum* f.sp. *cubense* race 1 (Figure 4.15). Its accession number is EU334870.1 and exhibiting *Fusarium oxysporum* f.sp. *cubense* biotype race1 (FPD1) gene, complete cds.

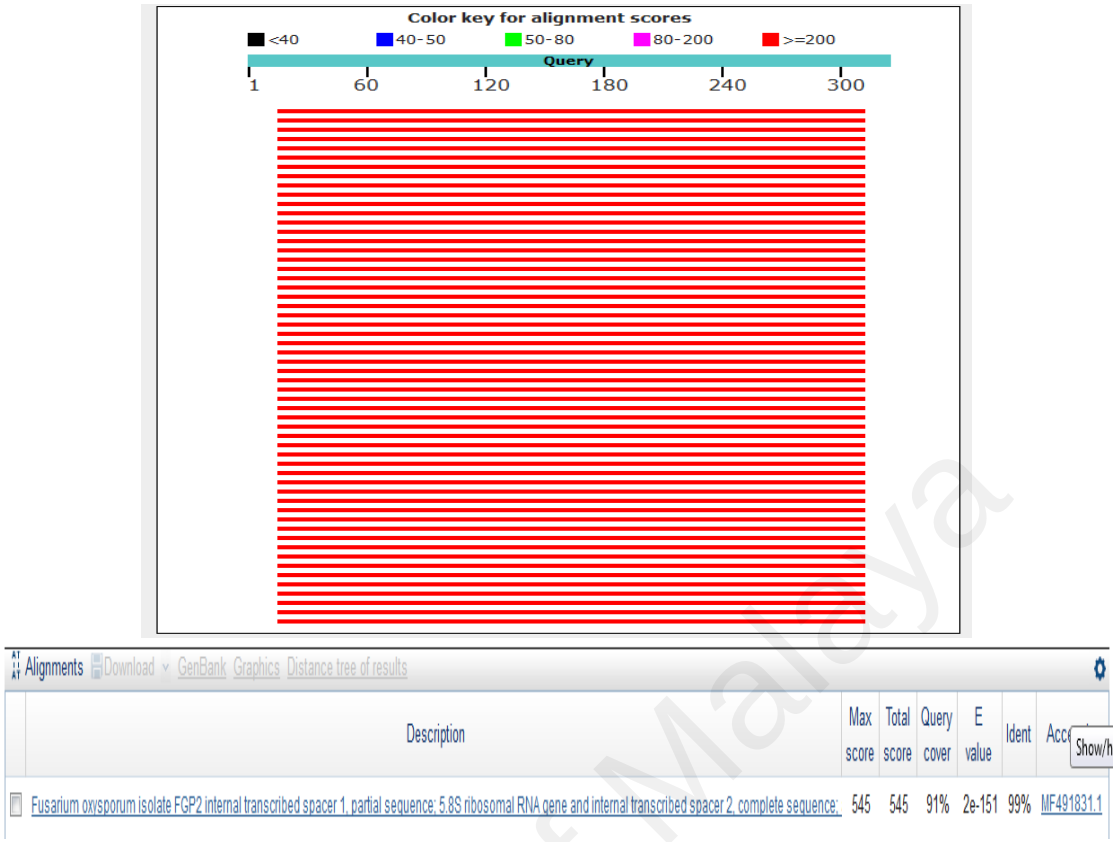


Figure 4.14: Sequence with highest nucleotide identity (99%) with fragment amplified using species specific primer sets.

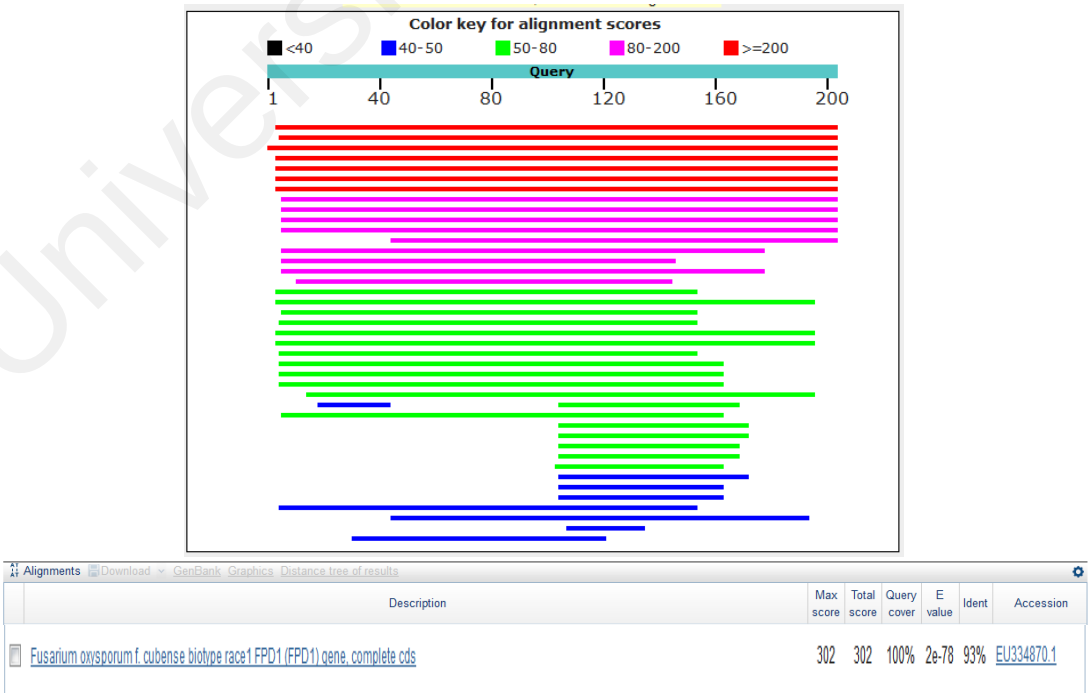


Figure 4.15: BLASTn analysis showed 93% similarity of amplified PCR fragment for race determination with *Foc* Race1 complete gene sequence.

4.5 Pathogenicity test

4.5.1 Acclimatization of banana plantlets

Rooted tissue cultured plantlets require special attention during the acclimatization process from the laboratory to a climate controlled greenhouse in order to avoid losses and ensure crop uniformity. As the laboratory is a relatively stress free environment, the plants were slowly phased into greenhouse conditions. This can be seen as the plantlet were slowly grow for the first two weeks in the greenhouse. On the third week and so on, they grew a bit faster and they start to develop 4 – 5 greenish leaves and well develop roots were observed on the eight weeks of hardening process.

4.5.2 Optimization of inoculation media

The growth of *Fusarium oxysporum* f. sp. *cubense* (C1HIR isolates) in two types of inoculation media, Potato Dextrose Broth (PDB) and Armstrong Liquid Media (ALM) were observedand shown in Table 4.5. By using the mean of each replicates, a graph were created to show the growth progression of *Foc* isolate C1HIR on both inoculation media tested (Figure 4. 16).

Based on the efficiency of PDB (DifcoTM, BD, France) as inoculation media to produce efficient result on the production of desired spore concentration, experiment were then continue to test the consistency performance of different Race 4 *Foc* isolates. Three selected Race 4 *Foc* isolates with positive morphology as *Fusarium* species tested on agar and further confirmed with molecular assay to confirm as *Foc* Race 4. All isolates were tested for its growth profile under same growth condition and spores concentration were counted on day 5 onwards (Table 4.6). Fermentation performance was shown in Figure 4.17. Maximal growth was observed on day 7^{th} of incubation for all three isolates tested.

Table 4.5: Spores concentration of C1HIR isolates (spores/ml) on different inoculation media tested.

Inoculation media		Day 5		Day 6		Day 7		Day 8		Day 9	
		Replicate	Avg	Replicate	Avg	Replicate	Avg	Replicate	Avg	Replicate	Avg
Armstrong's Liquid Media	1	$4.40x10^3$		$4.48x10^4$		$8.98x10^4$		$9.78x10^3$		$9.10x10^3$	
		$4.20x10^3$		$4.38x10^4$		$8.60x10^4$		$9.89x10^3$		$9.29x10^3$	
		$4.30x10^3$	$4.30x10^3$	$4.48x10^4$	$4.50x10^4$	$8.80x10^4$	$8.79x10^4$	$9.80x10^3$	$9.82x10^3$	$9.20x10^3$	$9.20x10^3$
	2	$4.26x10^3$		$4.41x10^4$		$8.00x10^4$		$6.54x10^4$		$6.45x10^3$	
		$4.18x10^3$		$4.41x10^4$		$8.10x10^4$		$6.54x10^4$		$6.40x10^3$	
		$4.10x10^3$	$4.18x10^3$	$4.50x10^4$	$4.44x10^4$	$8.09x10^4$	$8.06x10^4$	$6.59x10^4$	$6.54x10^4$	$6.46x10^3$	$6.44x10^3$
	3	$3.19x10^3$		$1.48x10^4$		$9.89x10^4$		$6.63x10^4$		$6.10x10^4$	
		$3.20x10^3$		$1.50x10^4$		$9.85x10^4$		$6.59x10^4$		$6.09x10^4$	
		$3.20x10^3$	$3.20x10^3$	$1.48x10^4$	$1.47x10^4$	$9.86x10^4$	$9.90x10^4$	$6.59x10^4$	$6.60x10^4$	$6.10x10^4$	$6.09x10^4$
Potato Dextrose Broth	1	$2.00x10^5$		$3.4\ x10^5$		$8.7\ x10^5$		$8.52x10^5$		$7.90x10^5$	
		$2.09x10^5$		$3.4\ x10^5$		$8.7\ x10^5$		$8.50x10^5$		$7.91x10^5$	
		$2.00x10^5$	$2.0\ x10^5$	$3.4\ x10^5$	$3.4\ x10^5$	$8.7\ x10^5$	$8.7\ x10^5$	$8.50x10^5$	$8.5\ x10^5$	$7.95x10^5$	$7.92x10^5$
	2	$4.48x10^4$		$4.80x10^4$		$9.08x10^5$		$9.70\ x10^5$		$9.52x10^5$	
		$4.50x10^4$		$4.88x10^4$		$9.19x10^5$		$9.75\ x10^5$		$9.53x10^5$	
		$4.49x10^4$	$4.49\ x10^4$	$4.80x10^4$	$4.83x10^4$	$9.08x10^5$	$9.08x10^5$	$9.76\ x10^5$	$9.74x10^5$	$9.53x10^5$	$9.53x10^5$
	3	$3.70x10^4$		$4.50x10^4$		$8.55x10^5$		$9.84\ x10^5$		$7.30x10^5$	
		$3.68x10^4$		$4.51x10^4$		$8.50x10^5$		$9.87\ x10^5$		$7.24x10^5$	
		$3.70x10^4$	$3.70\ x10^4$	$4.49x10^4$	$4.50x10^4$	$8.50x10^5$	$8.52x10^5$	$9.87\ x10^5$	$9.86x10^5$	$7.29x10^5$	$7.30x10^5$

Table 4.6: Spores concentration (spores/ml) of *Foc* Race 4 isolates produced using PDB (Difco™, BD, France) as inoculation media.

Sample	Day 5			Day 6		Day 7		Day 8		Day 9	
	Rep		Ave	Rep	Avg	Rep	Avg	Rep	Avg	Rep	Avg
C1HIR	1	$2.95x10^5$	$2.65x10^5$	$3.60x10^5$	$3.58x10^5$	$1.30x10^6$	$1.30x10^6$	$1.20x10^6$	$1.22x10^6$	$1.05x10^6$	$1.07x10^6$
	2	$2.50x10^5$		$3.75x10^5$		$1.29x10^6$		$1.25x10^6$		$1.05x10^6$	
	3	$2.50x10^5$		$3.40x10^5$		$1.30x10^6$		$1.20x10^6$		$1.10x10^6$	
9888	1	$3.05x10^5$	$3.02x10^5$	$4.30x10^5$	$4.32x10^5$	$9.83x10^5$	$9.88x10^5$	$9.50x10^5$	$9.47x10^5$	$7.85x10^5$	$7.79x10^5$
	2	$3.00x10^5$		$4.25x10^5$		$9.85x10^5$		$9.45x10^5$		$7.77x10^5$	
	3	$3.00x10^5$		$4.40x10^5$		$1.00x10^6$		$9.45x10^5$		$7.75x10^5$	
2296	1	$9.25x10^4$	$9.35x10^4$	$3.80x10^5$	$3.68x10^5$	$1.20x10^6$	$1.20x10^6$	$9.50x10^5$	$9.52x10^5$	$7.30x10^5$	$7.27x10^5$
	2	$9.35x10^4$		$3.50x10^5$		$1.21x10^6$		$9.50x10^5$		$7.25x10^5$	
	3	$9.35x10^4$		$3.75x10^5$		$1.20x10^6$		$9.55x10^5$		$7.25x10^5$	

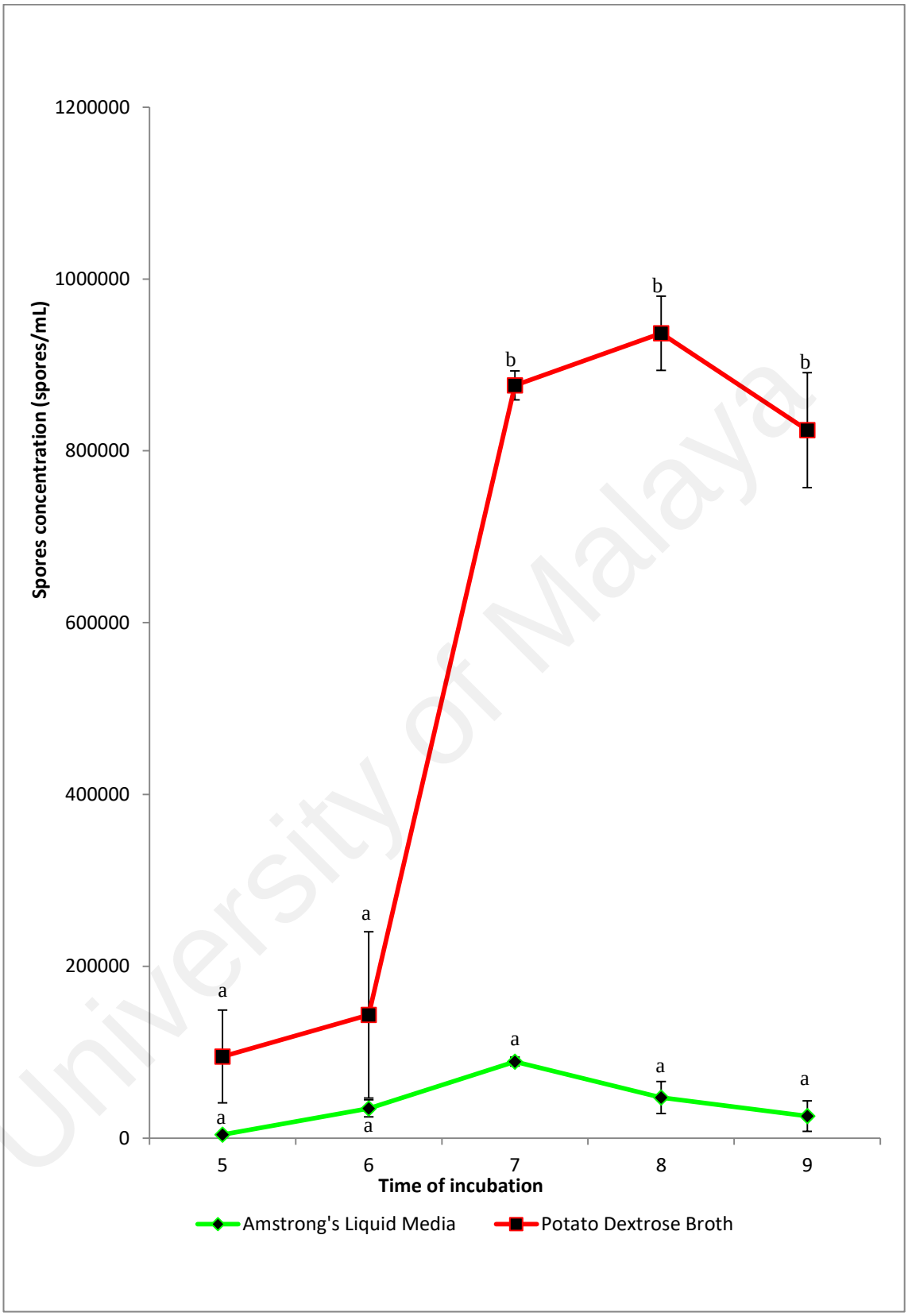


Figure 4.16: Comparison regarding growth progression of fungal culture *Foc* isolate C1HIR on different inoculation media. All data were subject to one way ANOVA. The data shown are expressed as mean (%) of three replicates + SE from three representative experiments. SE indicates standard error bar, same letter denotes not significant ($p>0.05$) by Duncan test.

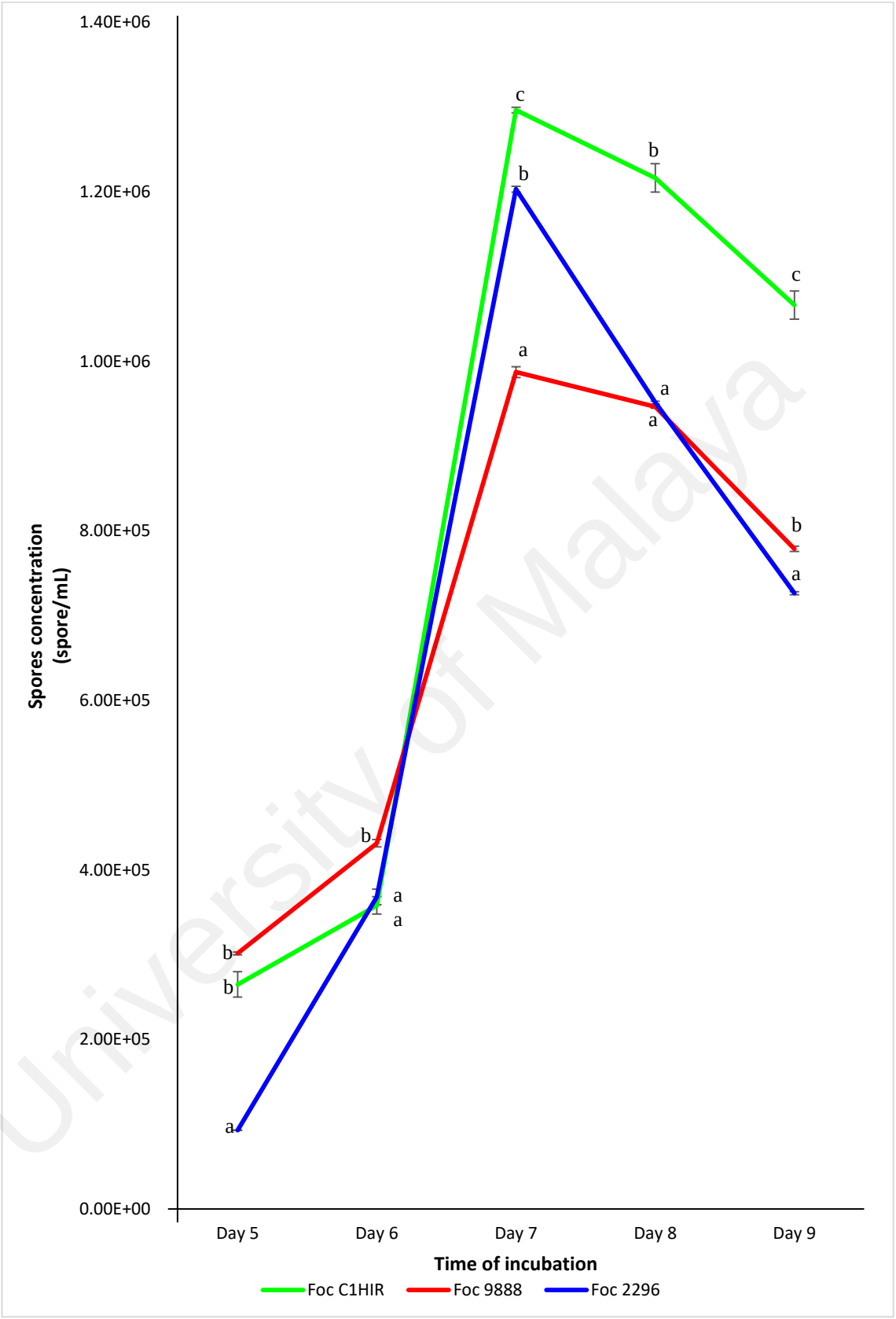


Figure 4.17: Spore growth of *Foc* Race 4 isolates in Potato Dextrose Media (PDB) inoculation media. All data were subject to one way ANOVA. The data shown are expressed as mean (%) of three replicates with SE from three representative experiments. SE indicates standard error bar, same letter denotes not significant ($p<0.05$) by Tukey HSD Multiple Comparison Test.

Spores production were also optimize in the shaking method. In this study, spores place in rotary shaker did not produce desired amount of spore (10^5-10^6 spores/mL) and even the suspension turned cloudy (Figure 4.18). The optimum and suitable growing condition for *Foc* isolates was through shaking manually twice per day in room temperature.

Figure 4.18: Fungal culture of *Foc* on PDB (Difco^{TM}, BD, France) media turned cloudy when incubated on rotary shaker at 60 rpm.

4.5.3 Bioassay challenge of *Musa acuminate* cv. 'Berangan' plants against *Foc*

Acclimatized tissue culture derived plantlets cv. Berangan were evaluated for disease response to *Foc*. The plantlets were infected with *Foc* inoculum as describe in section 3.5.5. C1HIR isolate was chosen as a model to construct an improve bioassay protocols for early screening of Fusarium wilt disease. The experiment was then repeat one time to ensure reproducibility. The same procedure and methods were then been used to evaluate disease response of cv. Berangan plantlets using the other 4 isolates of *Foc* with two isolates of race 1 (isolate 2306 and 10201) and race 4 (isolate 2296 and 9888) each. Table 4.7 and 4.8 show the computed DSI values for the yellowing and rhizome discoloration after 5 weeks of infection. Yellowing referred as Leaf Severity Index (LSI) while rhizome discoloration was referred as Rhizome Discoloration Index (RDI). Raw data for LSI and RDI scores were describe in Appendix C.

Table 4.7: Disease severity index for derived tissue culture *Musa acuminata* cv. 'Berangan' challenge with C1HIR isolates after 5th week of infection.

Isolate	DSI		Final Translation
C1HIR	LSI	RDI	
Trial 1	Highly susceptible	Susceptible	Highly susceptible
Trial 2	Highly susceptible	Susceptible	Highly susceptible

Table 4.8: Disease severity index recorded for other *Foc* isolates tested on derived tissue culture cv. 'Berangan' after 5th week of infection.

Isolate tested	DSI		Final Translation
	LSI	RDI	
Race 1(2306)	Susceptible	Tolerant	Susceptible
Race 1 (10201)	Highly susceptible	Susceptible	Highly susceptible
Race 4 (2296)	Highly susceptible	Susceptible	Highly susceptible
Race 4 (9888)	Highly susceptible	Highly susceptible	Highly susceptible

Plantlets challenged with 1 x 10^6 spores/ml per inoculation showed disease symptoms on the first plantlet as early in one week after being inoculated. Symptom was detected visually through external wilting and a light yellow coloring of the lower leaves. Yellowing symptom of the leaves will later progressed to the mid rib area. As the disease advance, more severe symptoms were observed mostly after 3 weeks of post-inoculation where more of the leaves become bright yellowing and die. Completely wilted leaves eventually fell off the petiole forming a 'skirt' of dead leaves surrounds the pseudo-stem (Figure 4.19A-D). Leaf discoloration Index (LSI) was measured based on 5 scores ranging from 1 to 5 with 5 as complete wilting of the leaf (Figure 4.20). Uninoculated plantlets did not show wilting symptoms due to *Foc* infection (Figure 4.19E-F).

Internal symptoms (rhizome discoloration), appeared as brownish to darkish discoloration at the junction of root and rhizome (Fig. 4.21A). Rhizome discoloration inde (RDI) was measured based on 8 scores ranging from 1 to 8 with 8 as complete discoloration observed inside the rhizome region (Figure 4.22). As the hyphae advanced

during the infection and colonized the vascular tissues, more discoloration was observed especially in the stellar region of the rhizome indicating a more severe disease progression (Figure 4.21B). Rhizomes of uninoculated plantlets used as a control with no discoloration observed as shown in Figure 4.21 C.

The yellowing DSI values for cv. 'Berangan' challenge with C1HIR was recorded at 3.74 indicated cv. 'Berangan' was highly susceptible while rhizome discoloration indicated susceptible response respectively (Table 4.7). Final disease evaluation was shown that cv. 'Berangan' was highly susceptible towards C1HIR isolates. The same response of cv. 'Berangan' was recorded in the second trial of infection assay using the same procedure and method for the same C1HIR isolates (Table 4.7).

Plantlets cv. 'Berangan' was susceptible for *Foc* Race 1 isolate 2306. The DSI for yellowing was at 3 while 2.30 for rhizome discoloration indicate that it is susceptible and tolerant towards isolate 2306 respectively (Table 4.8). The other isolate of *Foc* Race 1 tested was 10201 and this isolates gave different response towards cv. 'Berangan'. *Foc* 10201 isolate shown to cause highly severe response towards cv. 'Berangan' with DSI for yellowing and rhizome discoloration recorded were 4.00 and 4.21 respectively. Meanwhile, both Race 4 isolates tested shown consistent results. *Foc* Race 4 isolate 2296 shown DSI of yellowing of 3.22 and 4.52 for rhizome discoloration. Isolate 9888 of *Foc* Race 4 achieve DSI of 3.61 and 5.91 for yellowing and rhizome discoloration respectively. These results gain a final disease translation of highly susceptible indicate that cv. 'Berangan' response towards both isolates of *Foc* Race 4 were highly severe (Table 4.8). Details data recorded were attached in Appendix C section.

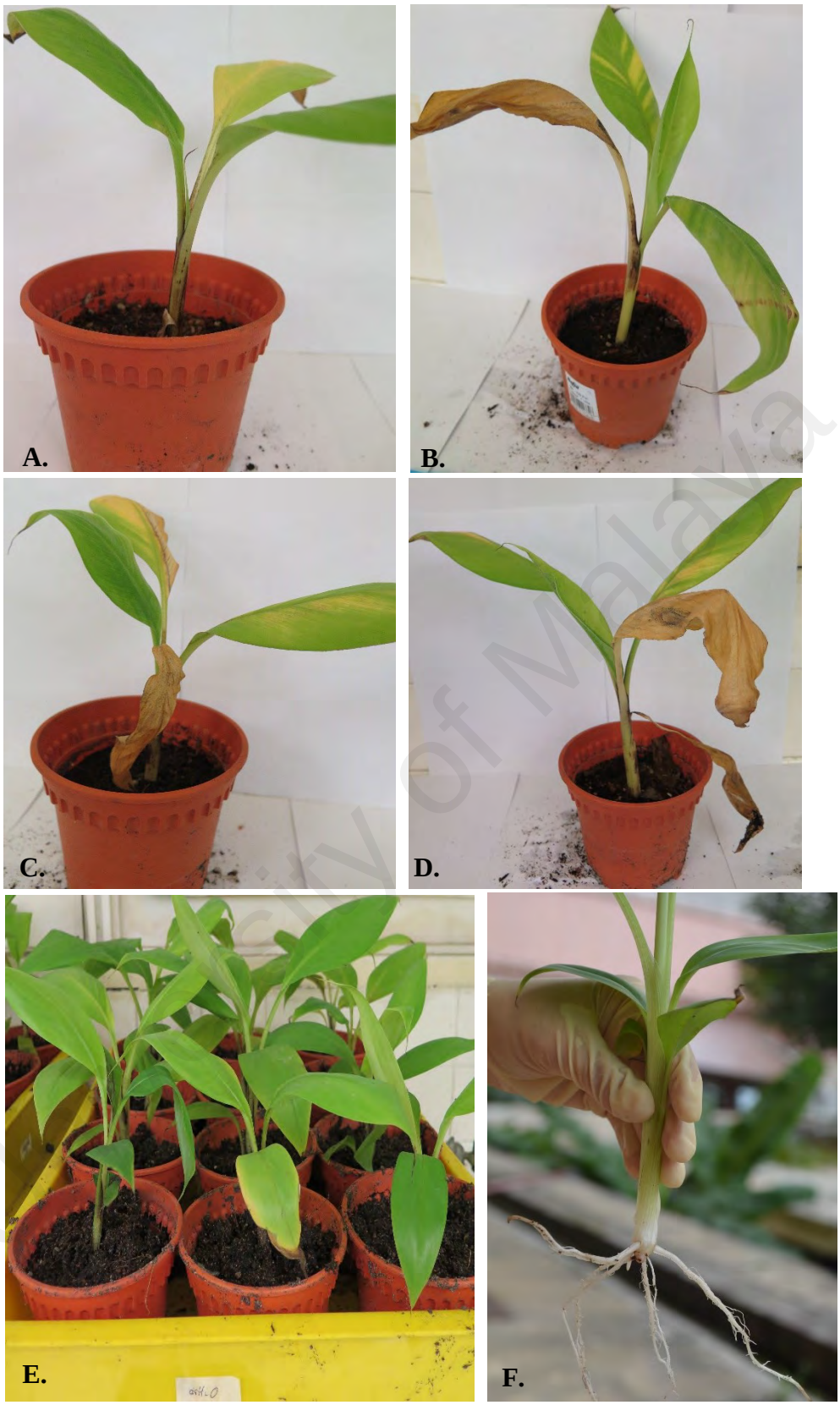


Figure 4.19: A: Plant showing early symptom of Fusarium wilt. B: Plant showing advanced symptoms of Fusarium wilt. C & D: Yellowing and dead leaves eventually collapse along the leaf stalk or at the junction of the stalk and stem, resulting in a skirt of dead leaves forming around the pseudo-stem. E: No streaking or yellowing of leaves detected on the uninoculated plants. F: Uprooted uninoculated plants appears healthy with whitish and vigorous roots.

Figure 4.20: Illustrated schematic diagram for LSI as reference for scoring purpose.

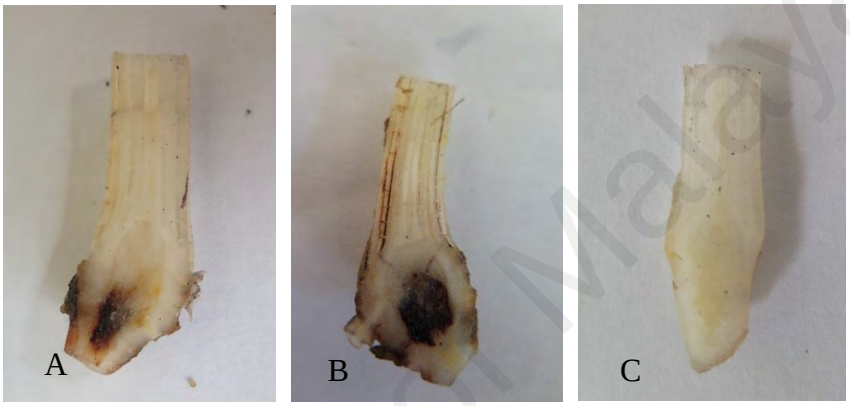


Figure 4.21: Internal symptoms of Fusarium wilt in cv. 'Berangan'. A; Longitudinal cut of rhizome showing discoloration at the junction of root and rhizome. B; Dark-brown discoloration in the stellar of rhizome region appear as the progress of disease caused by *Fusarium oxysporum* f. sp. *cubense* after 5 weeks of inoculation. C; Longitudinal section of non-inoculated plants of the same cultivars.

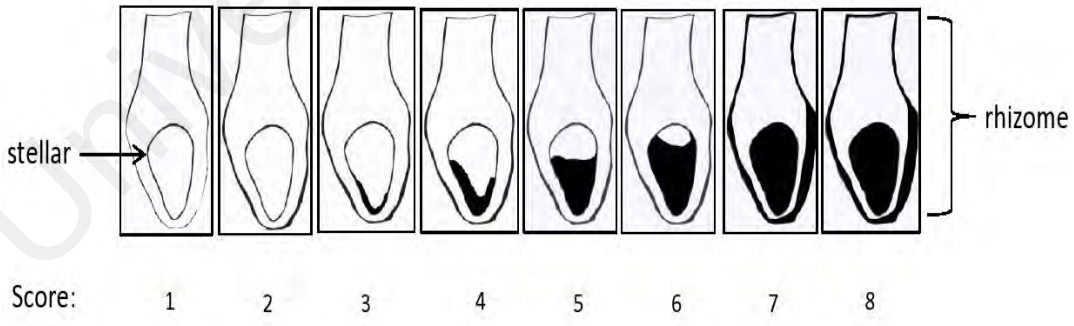


Figure 4.22: Illustrated schematic diagram for RDI as reference for scoring purpose.

4.5.4 Field Emission Scanning Electron Microscope (FESEM) observations

FESEM examination on thin section rhizome of Musa cv. 'Berangan' infected with *Fusarium oxysporum* f. sp. *cubense* (*Foc*). FESEM imaging revealed the presence of *Foc* microconidia and chlamydospore as early as 2 day of post-inoculation (Figure 4.23) on the sample surface.

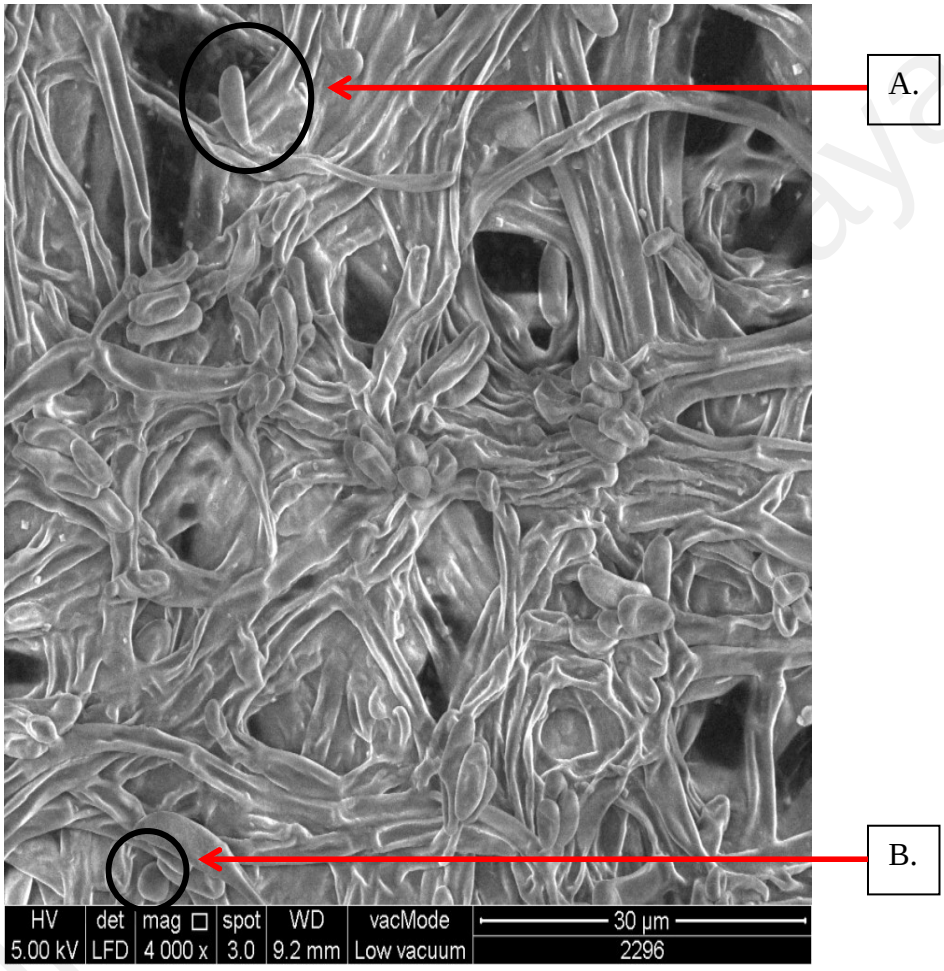


Figure 4.23: Scanning electron microscopy image of the mycelium of the fungus *Fusarium oxysporum* f. sp. *cubense*. The mycelium contains of a mass of branching structure, thread-like hyphae through which the fungus absorbs nutrients. (A) Microconidia growing from aerial mycelium. (B) A single, terminal chlamydospore.

4.6 Quantitative Reverse Transcriptase PCR

4.6.1 RNA quality and integrity

RNA purified from both inoculated and non-inoculated of banana roots was found to be of high quality. Electrophoresis of the total RNA of all roots samples showed the typical pattern with distinct 26S and 18S bands in the desired range of purity ($A_{260/280}$). The total RNA purity between 260:280 values must be in the range of 1.8 - 2.00 which signified good purity. All contamination of genomic DNA was completely remove for all cDNA samples following DNase treatment.

The results, presented in Table 4.9, shows that total RNA concentrations of all samples were found to be sufficient for real time PCR analysis and their purity (as indicated by the A260/A280) was found to be ideal for further analysis. The electrophoretic gels of all analyzed samples are also shown in Figure 4.24, 4.25 and 4.26.

Table 4.9: Quality control results for the total extracted RNA samples.

Sample	Concentration (ng/µl)	$A_{260/280}$ ratio
Control		
Day 0	122	1.900
Day 2	166	1.822
Day 4	150	1.900
Treated (Race 1)		
Day 0	125	1.893
Day 2	98	1.846
Day 4	137	1.882
Treated (Race 4)		
Day 0	161	1.800
Day 2	82.7	1.812
Day 4	176.9	1.824

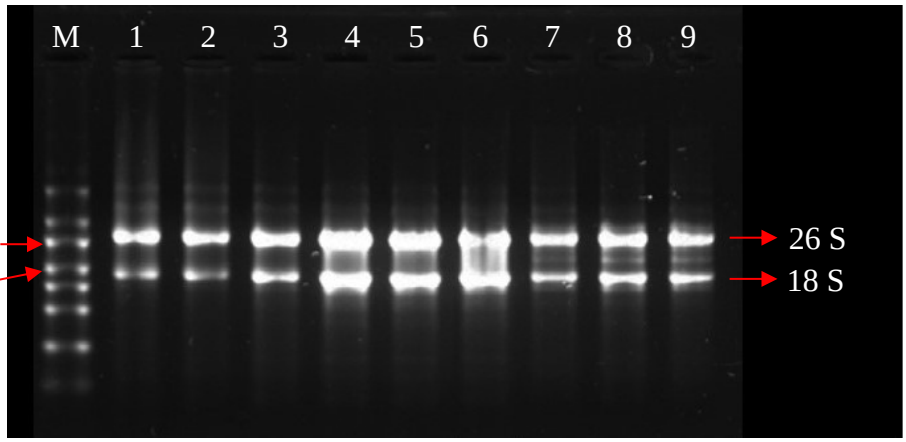


Figure 4.24: RNA integrity of control samples. M; High range RNA ladder (Promega), Lane 1-3; RNA extracted from sample Day 0 with triplicate, Lane 4-6; RNA extracted from samples Day 2, Lane 7-9; RNA extracted from samples Day 4.

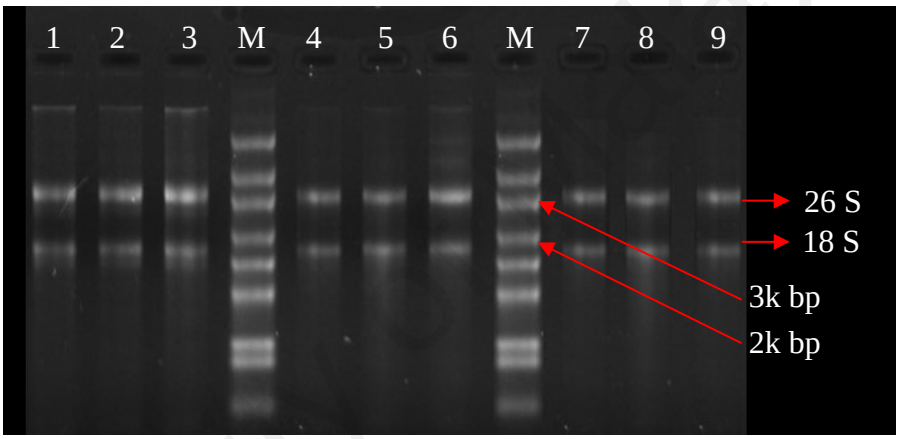


Figure 4.25: RNA integrity of samples inoculated with *Foc* Race 1 isolates (10201). M; High range RNA ladder (Promega), Lane 1-3; RNA extracted from sample Day 0 with triplicate, Lane 4-6; RNA extracted from samples Day 2, Lane 7-9; RNA extracted from samples Day 4.

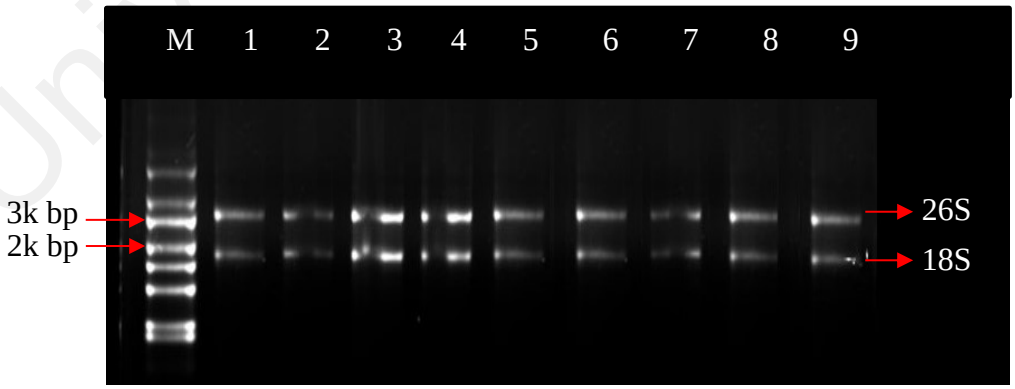


Figure 4.26: RNA integrity of samples inoculated with *Foc* Race 4 isolates (C1HIR). M; High range RNA ladder (Promega), Lane 1-3; RNA extracted from sample Day 0 with triplicate, Lane 4-6; RNA extracted from samples Day 2, Lane 7-9; RNA extracted from samples Day 4.

4.6.2 Standard curve

A standard curve of five serial two-fold dilutions of cDNA was used to determine the qPCR efficiency of each primer sets used. All PCR reactions displayed a correlation coefficient $R^2 > 0.98$ and efficiencies (E) > 99%. The results (cycle threshold or Ct values) were plotted against the $\log_{10}$ of each dilution factor. The efficiency obtained for all primers were greater than 99% indicating good primer specificity and amplification efficiency (Figure 4.27 – 4.30).

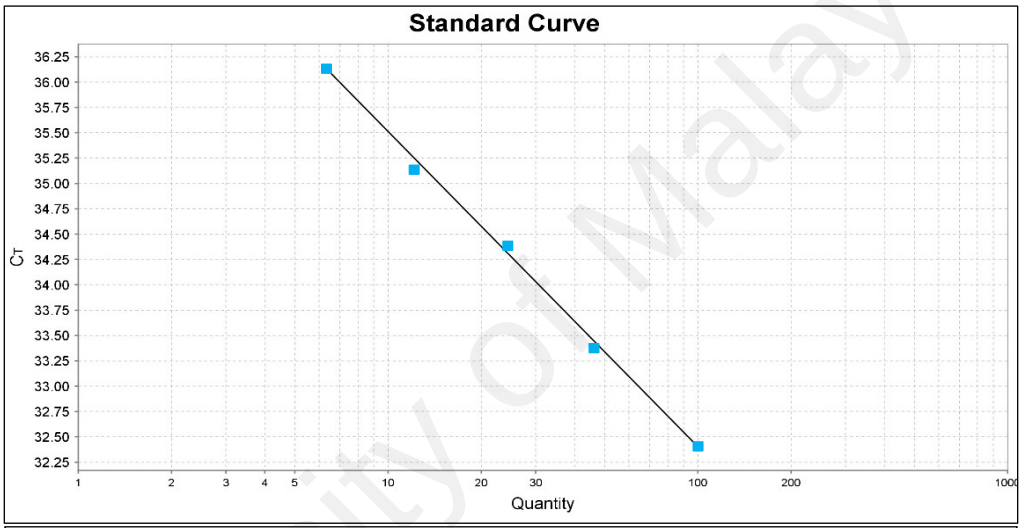


Figure 4.27: Standard curve for *GAPDH* gene for the real-time qPCR analysis of *Musa acuminata* cv. 'Berangan' infected with *Foc*.

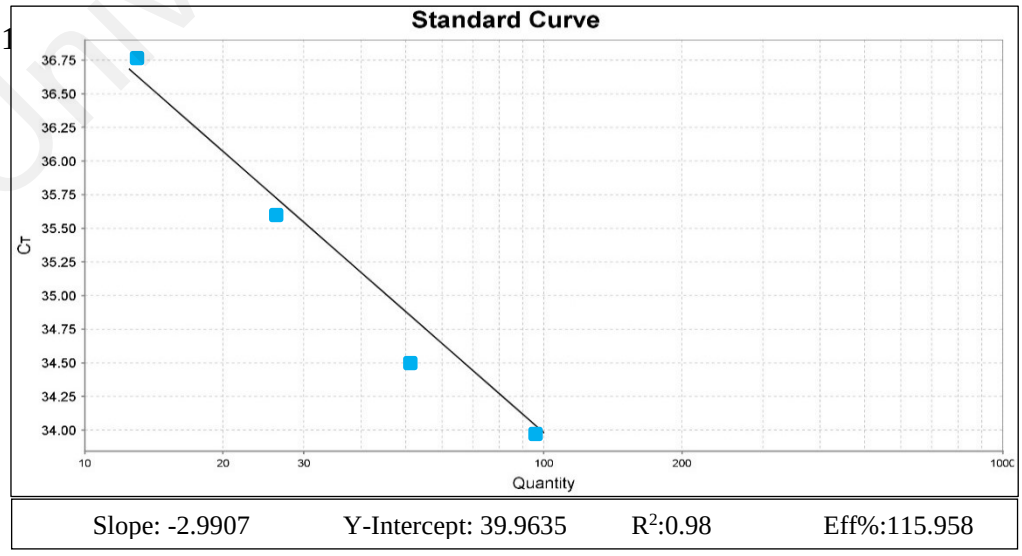
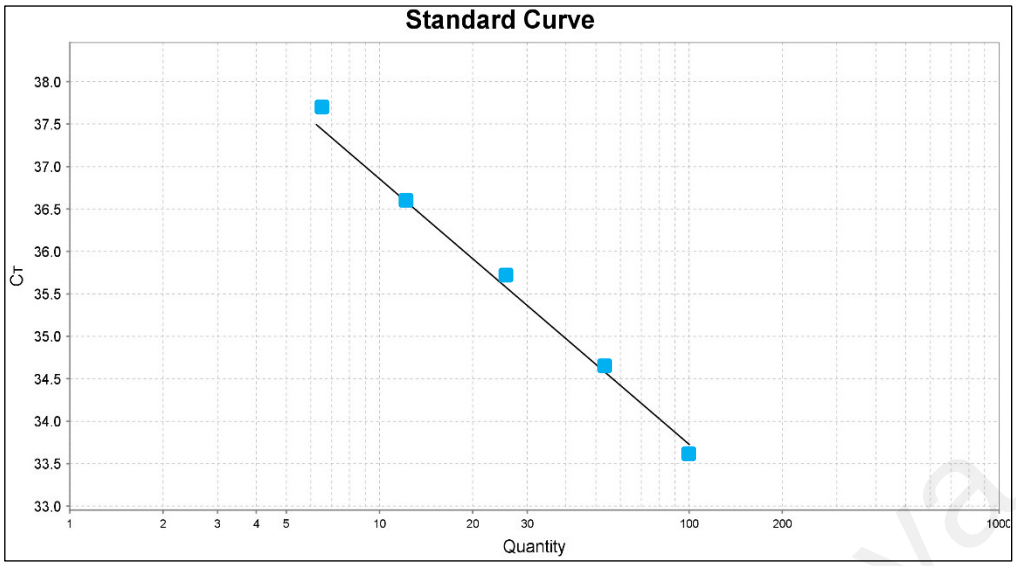
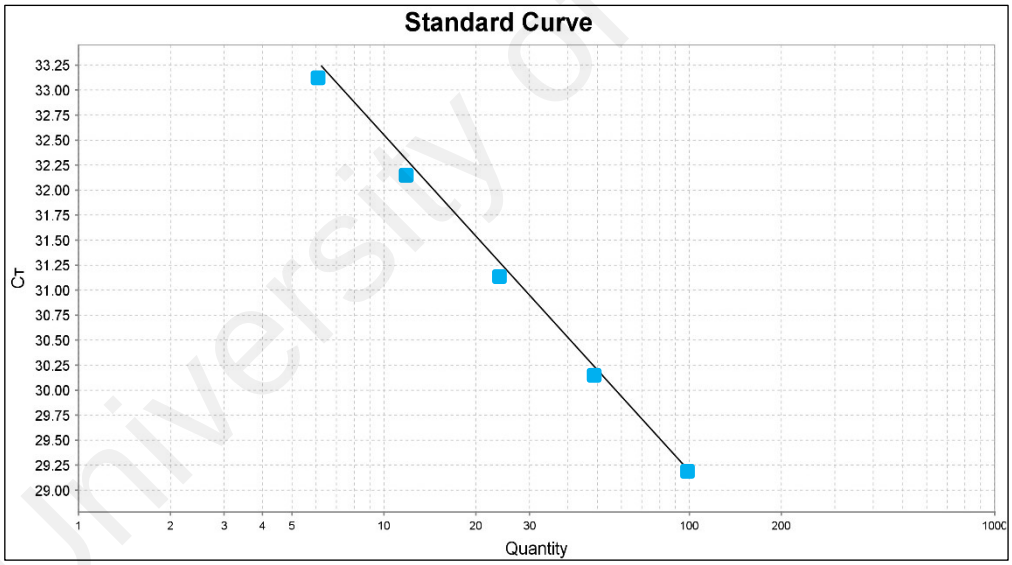


Figure 4.28: Standard curve for *RPS2* gene for the real-time qPCR analysis of *Musa acuminata* cv. 'Berangan' infected with *Foc*.



Slope: -3.1252	Y-Intercept: 39.9783	R^2:0.92	Eff%:108.92

Figure 4.29: Standard curve for *PAL* gene for the real-time qPCR analysis of *Musa acuminata* cv. 'Berangan' infected with *Foc*.



Slope: -3.361	Y-Intercept: 35.912	R^2:0.96	Eff%:99.399

Figure 4.30: Standard curve for *PR-10* gene for the real-time qPCR analysis of *Musa acuminata* cv. 'Berangan' infected with *Foc*.

4.6.3 Validation of reference genes

Based on the literature reports from *Musa* spp., two genes were selected as candidate reference genes. Average gene expression values and corresponding standard deviations were calculated for each gene from three repetitions for each assay run. The stability of mRNA expression for each of the 2 candidate reference genes was analyzed using raw data of Ct values and BestKeeper tools.

Using data obtained from the analysis, the stable reference gene were selected following two principles:

i. Genes that expressed constitutively over the time course of the *Foc*-banana interaction and
ii. Gene expression showing a low values of standard deviation for all time points examined.

The descriptive statistic of the Ct values and BestKeeper (Pfaffl et al., 2004) tool across all the samples of each gene are available in supplementary information (Appendix D). A line chart were then plotted for both reference genes using the Ct value (Figure 4.31 and 4.32) to see the pattern of expression for each gene.

Samples inoculated with *Foc* Race 4 at day 2 of post inoculation shows higher Ct value for *GAPDH* gene compared to day 0 of post-inoculation. Large fluctuation of the Ct value shows that the expression of *GAPDH* gene was not uniform in highly susceptible Musa cv. 'Berangan' following inoculation with pathogenic strain of *Foc*. *RPS 2* gene shows a less variable in Ct value indicating that this gene is more stable under experimental condition used herein. Calculation of standard deviation (Table C.1 & C.2) used to tell how Ct value in each gene express are spread out the average mean. Results show that standard deviation of *RPS 2* gene is lower compare to *GAPDH* gene. Low standard

deviation of *RPS 2* indicate that most of the Ct values are very close to the average Ct while high standard deviation of *GAPDH* denote that the Ct values are spread out.

In the BestKeeper evaluation, *RPS 2* has low stability value while *GAPDH* has high stability value (Figure 4.33). In this algorithm, the genes exhibit the lowest expression of stability values and smaller SD value indicate the most stable reference genes. *GAPDH* exceeded the standard deviation (SD) threshold value (more than 1.5) across all samples in this study, thus they were regarded as not suitable for gene expression normalization.

Overall, by using both method of analytical approaches, *RPS 2* can be concluded as the most suitable gene to be used as reference genes, so-called housekeeping gene for an accurate normalization to be performed.

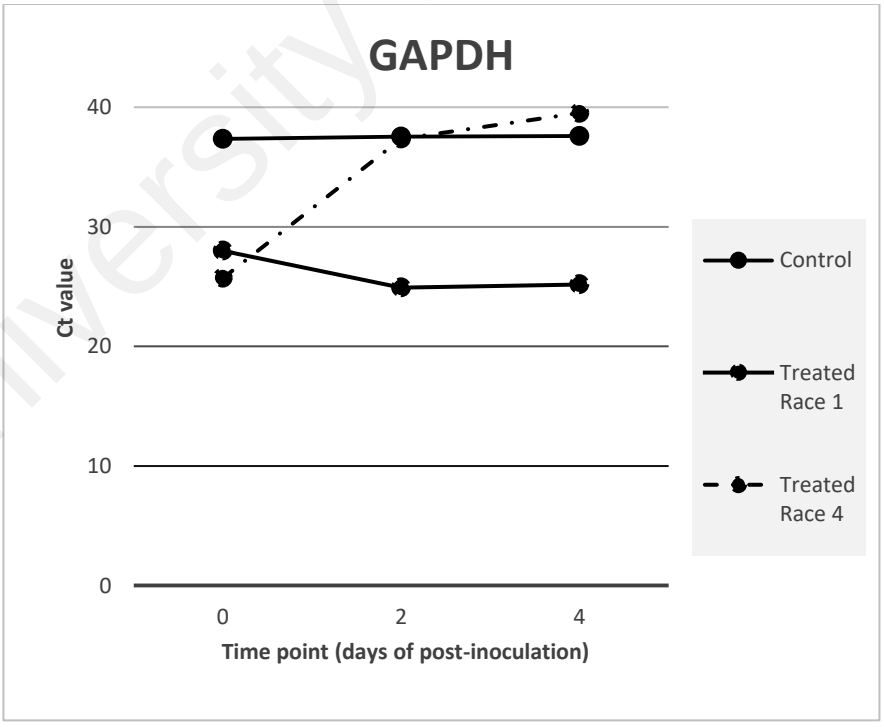


Figure 4.31: Pattern of expression for *GAPDH* gene tested under experimental condition used herein.

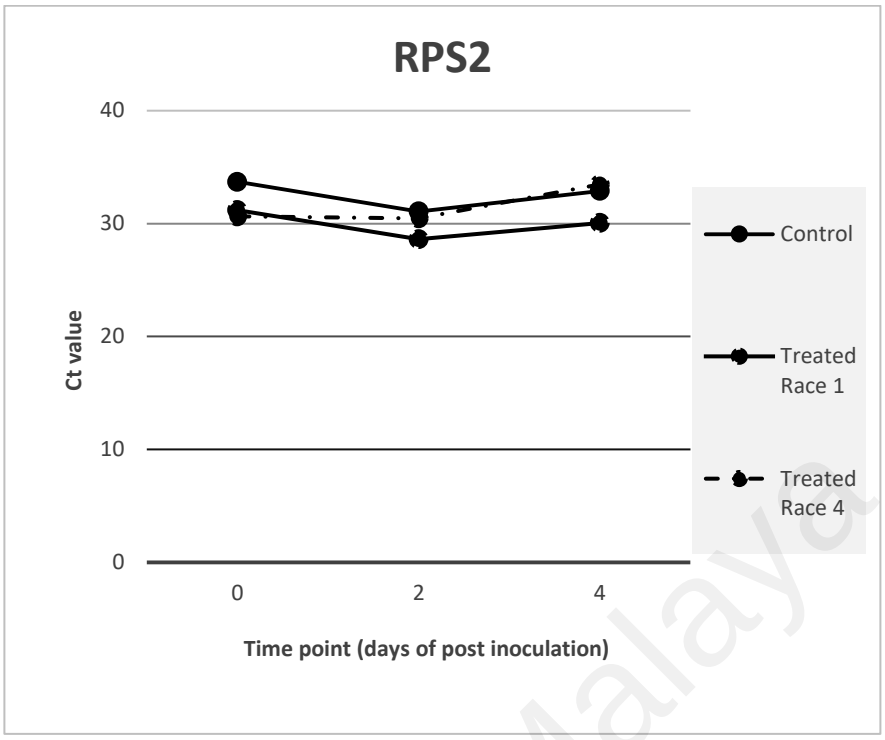


Figure 4.32: Pattern of expression for *RPS 2* gene tested under experimental condition used herein.

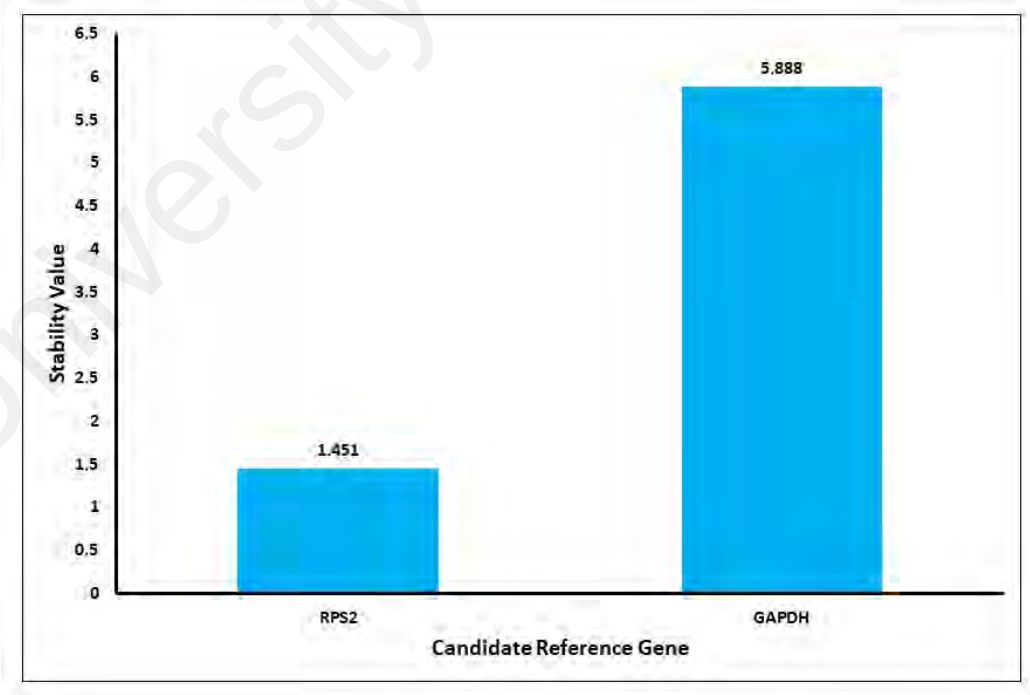


Figure 4.33: Evaluation of the expression stability for two candidate reference genes in highly susceptible *Musa acuminata* cv. 'Berangan' using BestKeeper tool. The most stable reference gene has the lowest expression stability values.

4.6.4 Analysis of defense-response genes expression during *Foc*-banana interaction

Expression of the defense-response genes were normalized using *RPS2* as endogenous control genes and the double delta Ct value ($\Delta\Delta C_T$) were calculated to compute fold change of gene expression (Table 4.10 and 4.11). Both defense-response genes revealed significant expression within 4 days of post-inoculation. Increased expression was detected for both genes (Figure 4.34 and 4.35).

Table 4.10: Double Delta Ct for defense-related gene for sample inoculated with *Foc* Race 1 isolates (10201). [2]

Target gene	Avg Treated Ct value		Avg Control Ct value		ΔCt Treated (DRG-HKG)	ΔCt Control (DRG-HKG)	Delta Delta Ct (ΔΔCt) (ΔCtT-ΔCtC)	Expression Fold Change (2^-ΔΔCt)	Log2 Fold
	DRG	HKG	DRG	HKG					
PR10									
Day 0	26.86	31.19	34.85	33.69	-4.33	1.16	-5.49	44.94	5.49
Day 2	23.72	28.62	32.86	31.08	-4.91	1.79	-6.69	103.58	6.69
Day 4	23.45	30.06	35.49	32.89	-6.61	2.60	-9.20	590.86	9.21
PAL									
Day 0	30.08	31.19	35.09	33.69	-1.11	1.40	-2.51	5.68	2.51
Day 2	27.73	28.62	33.26	31.08	-0.89	2.18	-3.07	8.43	3.07
Day 4	28.35	30.06	36.02	32.89	-1.72	3.13	-4.85	28.80	4.85

[2] Legend: Avg: Average; DRG: Defense-related Gene; HKG: House-keeping Gene.

Table 4.11: Double Delta Ct for defense-related gene for sample inoculated with *Foc* Race 4 isolates (C1HIR). [3]

Target gene	Avg Treated Ct value		Avg Control Ct value		ΔCt Treated (DRG-HKG)	ΔCt Control (DRG-HKG)	Delta Delta Ct (ΔΔCt) (ΔCtT-ΔCtC)	Expression Fold Change (2^-ΔΔCt)	Log2 Fold
	DRG	HKG	DRG	HKG					
PR10									
Day 0	24.62	30.63	34.85	33.69	-6.01	1.16	-7.17	144.14	7.17
Day 2	33.07	30.46	32.86	31.08	2.61	1.79	0.82	0.57	-0.82
Day 4	33.09	33.47	35.49	32.89	-0.39	2.60	-2.98	7.90	2.98
PAL									
Day 0	28.79	30.63	35.09	33.69	-1.84	1.40	-3.23	9.38	3.23
Day 2	34.18	30.46	33.26	31.08	3.71	2.18	1.53	0.35	-1.53
Day 4	36.63	33.47	36.02	32.89	3.16	3.13	0.02	0.98	-0.02

[3] Legend: Avg: Average; DRG: Defense-related Gene; HKG: House-Keeping Gene.

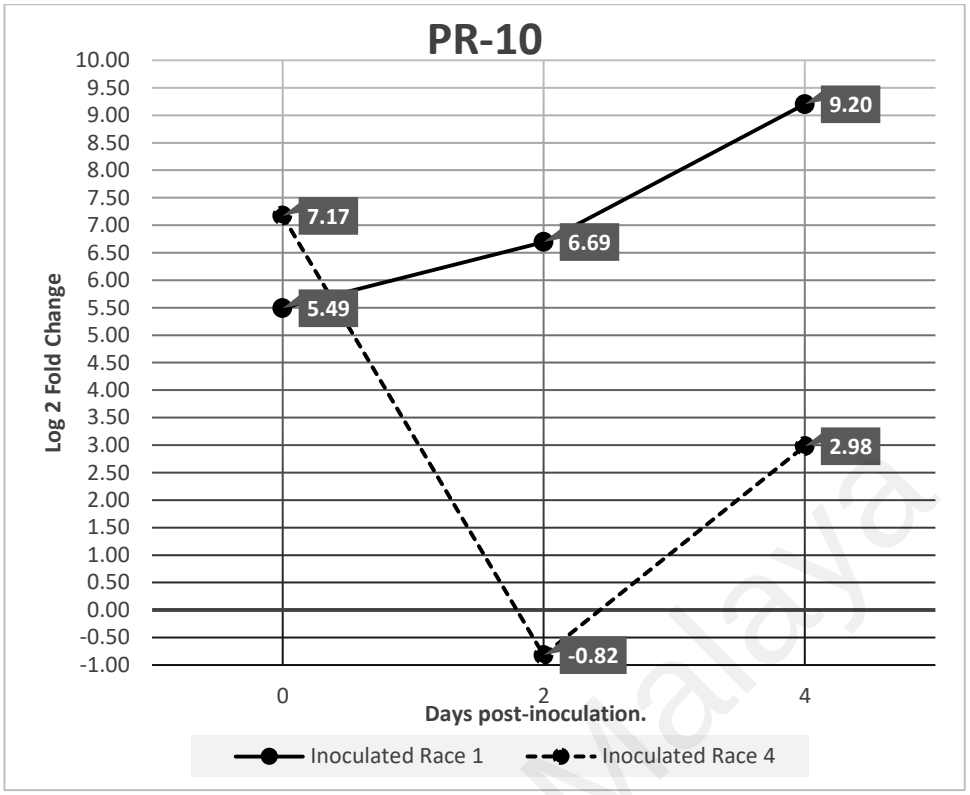


Figure 4.34: Expression of *PR-10* gene in *Musa acuminata* cv. 'Berangan' challenged with *Foc* Race 1 and Race 4 across three time points.

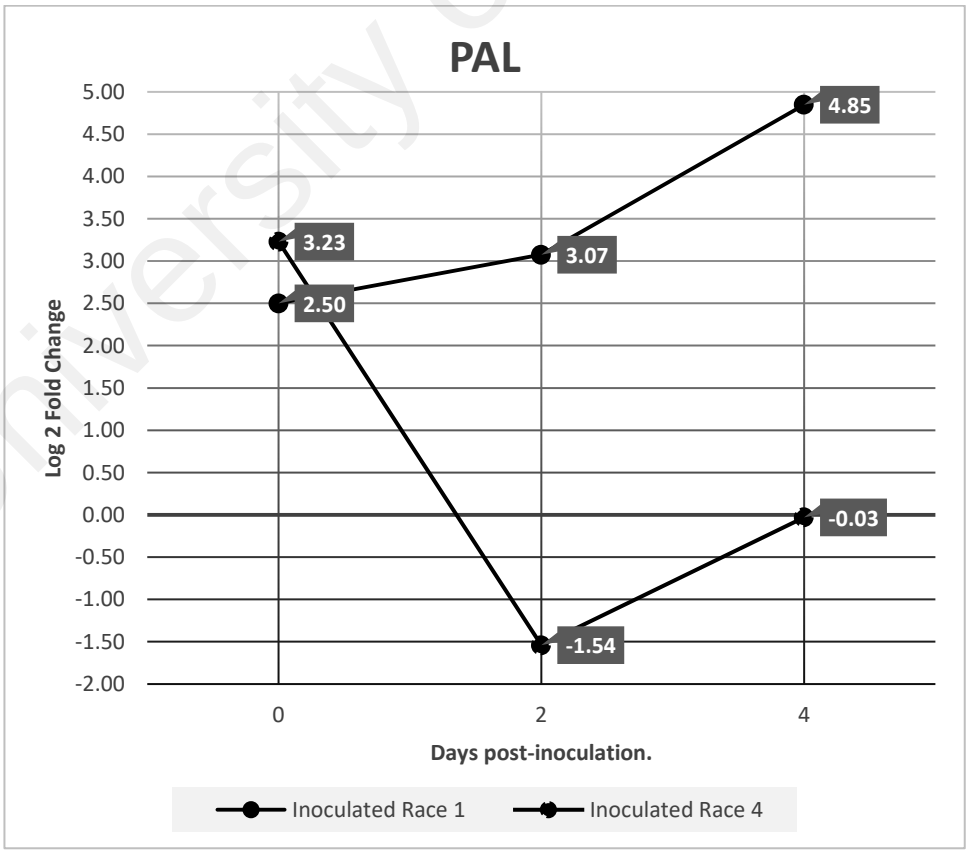


Figure 4.35: Expression of *PAL* gene in *Musa acuminata* cv. 'Berangan' challenged with *Foc* Race 1 and Race 4 across three time points.

Line charts were generated to present the differential expressions of the *PR-10* and *PAL* genes at different time points after inoculation with *Foc*. The chart shows interesting expression when inoculated with both *Foc* Race 1 and Race 4. The *PR-10* gene shows upregulated expressions throughout the Race 1 inoculation. These expressions show a 0.21 upregulated fold change on day 2 and further increase to 0.36 on day 4 (Figure 4.34). Not the same could be said for challenges done with the Race 4 isolate, the gene showed momentous down-regulated expression to -1.11 fold change on day 2 and then up-regulated to 4.63 on day 4.

When observing *PAL* gene expressions post-inoculation, the results are 0.23 folds on day 2 when challenged with the *Foc* Race 1 (Figure 4.35). The expressions were further upregulated on day 4 with fold change of 0.57. When inoculated with the *Foc* Race 4, *PAL* gene expressions significantly down regulated to -1.47 fold change on day 2. Interestingly, gene expressions were then up regulated with 0.98 fold change on day 4.

CHAPTER 5: DISCUSSION

5.1 Morphological characterization of *Fusarium*

The morphological characteristics were studied in forty-two fungal isolates collected from the banana plantations regions of all around Malaysia (Chapter 3.1.1). Results (Table 4.1) revealed that all the forty-two representative isolates of *Fusarium* exhibited a great variability in respect of colony characteristic and pigmentation colour.

In this study, it was sometimes difficult to distinguish *F. oxysporum* from other species of *Fusarium* based solely on the morphological features. This is similar with the conclusions by Gupta & Ayyachamy, (2012). Thus, molecular characterization was conducted for confirmatory identification of *F. oxysporum* using PCR analysis. Based on the morphological identification, the *Fusarium* species identified were *F. solani* (4 isolates), *F. oxysporum* (31 isolates) and other *Fusarium* spp (7 isolates). Based on the literature studied, *F. solani* and *F. oxysporum* were the most frequently isolated and the most common species associated with Fusarium wilt symptoms (Hafizi et al., 2013).

Colony appearances of *F. oxysporum* as observed on PDA (Difco™, BD, France) were greatly variable (Nelson et al., 1983). Fungal culture of *F. oysporum* grew fast on PDA (Difco™, BD, France) and the prominent mycelia appeared clearly within 3 days of culture. Mycelia observed were described as off white in color, fairly dense and display pigmentation at the periphery region on the plates. The peach-violet colony colour and pigmentations observed in some isolates of *F. oxysporum* were consistent with the description clarify by Booth, (1971). Some other isolates culture showing bright pink pigmentation at the center region, and some had creamy yellowish pigmentation. The light pink and purple pigmentation was also observed on PDA (Difco™, BD, France) by other researchers also (Faganello et al., 2017; Ignjatov et al., 2012; Pérez-vicente et al.,

2014). The pattern of mycelia growth was in thick concentric ring arrangement. Therefore, in this present study, *F. oxysporum* isolates could clearly be identified based on morphological characteristic and confirm the presence of *Fusarium spp.*

5.2 Molecular characterization of *Foc*

Development of specific primers for *Foc* was reported by other workers (Li et al., 2013; Waalwijk et al., 2011). Hence, the results described here proved that the primer pairs were suitable for a fast, reliable and specific identification of *Foc* isolates and could be suitable for early diagnosis of Fusarium wilt of banana by plant pathologist.

A total of 7 isolates of *F. oxysporum* were detected as *Foc* race 1 as it were successfully amplified 245 bp size of amplicon. Our current results shows that, *Foc* Race 1 falls into VCG 1217 and 1218. Some *Foc* Race 1 isolates were not compatible to lineage 6 VCGs.

The IGS region of 31 isolates of *F. oxysporum* were successfully amplified using PCR approaches for TR4 detection and the size of amplicon was approximately 452 bp. Out of 31 isolates of *F. oxysporum* tested, 17 isolates were positive for TR4. The results obtained in the present study demonstrate that all isolates of TR4 detected fell into VCG 01213/16 but not all VCG 01212/16 were TR4.

To date, TR4 belongs to a single group of VCG namely as VCG 01213. TR4 with VCG 01213/16 complex has also been reported by other researchers but most evidence stated that it is the same group as VCG 01213 (Dita et al., 2010). Molina et al. (2011) also revealed that Malaysian's *Foc* belongs to the VCG 01213/16 and it's associated with *Foc* TR4.

5.3 Field sampling of vascular tissue colonization in Jeli, Kelantan

Yellow and wilted leaves are typical symptoms of Fusarium wilt disease. Based on the observation in the field, the yellowing typically progresses from the older to the younger leaves. The wilted leaves may remain erect or collapse at the petiole and hang down along the pseudostem (Figure 4.4). The external symptom of Fusarium wilt can be confused with those of the bacterial disease Xanthomonas wilt. In plants that were infected by Xanthomonas, the wilting can begin with any leaves and tend to snap along the leaf blade. Furthermore, cream to pale yellow bacterial ooze will appears on the cutting of the pseudostem (Borkar & Yumlembam, 2016).

The pseudostem sections of infected plant showed the symptoms of panama wilt in the form of brown discoloration of the vascular bundle (Groenewald et al., 2006). In our study, the discoloration of the pseudostem was seen in the cutting of the pseudostem and it's in a ring pattern (Figure 4.4B). This is due to the fact that, the *Fusarium* species are soil borne pathogens, which attack the water conducting vessels of the banana plants (Okungbowa & Shittu, 2012). In contrast to the bacterial wilt causing Moko disease, the discoloration ranges from pale yellow to dark brown, and the discoloration could be found within the central portion of the pseudostem (Alvarez, et al., 2015).

Proposed by most papers (Aguayo et al., 2017; Carlier et al., 2003; Dita et al., 2010; Pérez-vicente et al., 2014), the vascular tissue samples were dissected from the specimen as soon as it was collected. The aseptic technique must be practiced throughout the dissection process. Coloured vascular strands were placed on a sterile filter paper in the petri plates. These plates were then placed under a laminar flow (Figure 4.5). This is to prohibit the growth of bacteria that could complicate the isolation process (Pérez-vicente et al., 2014). These strands should be dried for a few days, and dehydration should be one

under natural conditions. It took 35 hours to dry the strands with a laminar flow. The flow is to enforce sterile conditions throughout the isolation process.

Isolation of the *F. oxysporum* were attempted as soon as the strands dried. PDA (Difco^{TM}, BD, France) was used as the medium in the isolation process as it is the most widely used medium in fungal isolation and culture. Other media such as malt dextrose agar, potato sucrose agar, and Czapek yeast autolysate agar were also commonly used, but they were ineffective in inducing sporulation of sterile isolates (Guo et al., 1998; Li et al., 2007; Su et al., 2012). According to Demain et al. (2000), sporulation generally occurs when the rate of the fungal growth is reduced and is hampered under circumstance that favor speedy mycelial growth. Depletion of nutrition usually stimulates sporulation process, and some modification of media with low nutrient have been applied (Braun et al., 2011; Wulandari et al., 2009). Thus in our study, we used quarter strength PDA (Difco^{TM}, BD, France) as low nutrient media, and Masangkay et al. (2000) revealed that, some typical low nutrient media include water agar, half or quarter strength PDA (DifcoTM, BD, France) and synthetic nutrient-poor agar medium. Previous work by Segalin et al. (2010) also revealed that quarter strength of PDA (Difco^{TM}, BD, France) was able to produce the highest radial growth of fungal, compared to Nash & Snyder agar (NSA) and Segalin-Reis agar (SRA-FG) for detection of *Fusarium* isolates. They also emphasize that, PDA (Difco^{TM}, BD, France) medium able to maintain the colony morphology of the fungal. Standard culture media such as PDA (Difco^{TM}, BD, France) support the growth of numerous fungal species plus often require particular pretreatment of the samples, and errors in such procedures can compromise the investigation validity due to the occurrence of undesirable contaminant (Segalin & Reis, 2010). To encounter these issue, samples in our research was surface sterilize with 70% ethanol (v/v) solution and samples were placed on sterile filter papers (Section 3.2.5.1).

In our study, we increased the streptomycin concentration to three fold in order to inhibit the growth of bacteria. Initially we used streptomycin with a concentration of 10g/L. Growth of the fungus was badly interfered by bacteria. Pérez-vicente et al. (2014) also brought this factor forward. Thus, we increased the concentration from 1.2 ml to 3.6ml for every 240 ml of PDA (DifcoTM, BD, France) of streptomycin. The technical manual had also recommended to let the samples dry further and increase the strength of the antibacterial amendment in the medium used.

5.3.1 Molecular analysis of symptomatic sample from farmer's field in Jeli, Kelantan

Fusarium isolates were successfully recovered from symptomatic rhizome and pseudostem of field samples assayed on PDA (Difco^{TM}, BD, France) medium. High quality of genomic DNA (gDNA) was obtained (Figure 4.9 and Table 4.4). All primers and amplification conditions resulted in high quality of amplicon of targeted genes (Figure 4.10, 4.11 and 4.12).

An amplicon size approximately 340 bp was successfully amplified from all symptomatic samples using primer pair of species specific for *Fusarium oxysporum* detection. All symptomatic samples tested were consistently produced the desired band as assay using 1% (v/v) agarose gel.

Race specific PCR assay were used for the specific detection of *Foc*'s race with race 1 and 4 specific primer pairs amplified a single band of 245 and 452 bp DNA fragments respectively. PCR using primer pair *Foc*-Race1-F/*Foc*-Race1-R were optimize with gradient PCR as the first amplification results show multiple bands were obtain (data not shown). Based on our results, we able to get suitable melting point for the amplification

of the primer pair, as single band we obtain using 53 to 58℃. Thus, by using this primer pair for race 1 detection of *Foc*, we proposed an annealing temperature of 53℃ as an ideal temperature using PCR approaches for a rapid and reliable detection method. However, when using the *Foc*-TR4-F/*Foc*-TR4-R primer pair for race 4 detection, no amplicon were amplified, thus confirming the race specific detection as our symptomatic samples are *Foc* race 1 and further analysis for verification was perform by sequencing analysis.

5.3.2 Sequencing analysis

The gene sequences from sequenced data were aligned and compared to available sequences deposited in the NCBI website to further confirm the identity of the *Foc* isolates. We used a BLASTn tools analysis of ITS rDNA sequence on the NCBI website as it was mentioned by Chen et al. (2015) that BLASTn tools exhibits great performance over MegaBLAST with faster computational speed. The alignment results are depicted in Figure 4.13. They indicated that the PCR amplified product for *Fo* identification yielded sequence identities with a similarity of 99% with the sequences of *Fusarium oxysporum* isolate. Its GenBank accession number MF491831 exhibited FGP2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence, and large subunit ribosomal RNA gene, partial sequence.

For race specific detection, the blasted results showed 93% sequenced similarity with *Fusarium oxysporum* f. *cubense* biotype race 1 *FPD1* (*FPD1*) gene, complete cds with an accession number EU334870.1.

Thus, we can conclude that our symptomatic sample from farmer's field in Jeli, Kelantan belongs to *Foc* race 1 and we would like recommend this procedures as a fast,

reliable and precise detection method for identification of *Foc* from symptomatic sample of banana plant. Furthermore, identification of the species, formae specialis and race levels is highly desired (Lievens et al., 2008), particularly for quarantine pathogens that are of high economic important (Dita et al., 2010).

5.4 Pathogenicity test

The continuing threat of *Fusarium* infection in banana worldwide has resulted in attempts to control the disease that has met with limited success. To date the development of resistant and tolerant varieties remains the most effective approach to overcome the problem. The proliferation of reports assessing the response of different banana to the pathogen using an array of next generation molecular tools have highlighted the need for a standardized bioassay to assess the performance of new varieties of banana toward *Fusarium* infection.

In pathogenicity test, results indicated that most of *F. oxysporum* in our collection used were pathogenic. These isolates caused wilt symptoms on cv. 'Berangan' tested.

5.4.1 Acclimatization of banana plantlets

In this experiment, disease-free tissue culture banana plantlet cv. 'Berangan' was used for the infection assay. Plantlet went through acclimatization process for two month prior to the infection. During the acclimatization process, caps of tissue culture jars were removed partially for a day or two which then were completely removed. During this stage, any contamination towards the agar medium are generally minor unless plantlets remained exposed to the open for more than a week (Ehirim et al., 2014). Gabr et al. (2010) called this process as pre-acclimatization process and he emphasize that this step

was important for better micro-propagation. Plantlets grown in the lab with optimum environments (moisture, salts, sucrose and water) lack cuticle layers in the leaves and also had higher rate of transpiration.

Plants were the deflasked from their culture jars and the agar residues were removed using slow running tap water. This is for the sucrose and nutrient in the agar (stuck in the plantlets) may serve as a medium for the growth of the organism that will cause any potential disease. Slow running tap water during the washing step is to avoid destroying the plantlets which during this stage of development, the plantlets tissues are still tender (Ehirim et al., 2014). Plantlets were then transferred onto soil and underwent acclimatization or hardening process for two months in a greenhouse.

Acclimatized plantlets are better able to survive and promote successful development of the plant. Tissue cultures raised plantlets usually develop a distinct morphology in response to the in vitro atmosphere in which they have been grown (Hiren, 2004). The difficulties associated with tissue culture raised plantlets are on the basis on the lack of water loss control from the plant through evaporation and their requirement to shift from heterotrophic to photoautotrophic nutrition (Ehirim et al., 2014). Lavanya and co-researchers (2009) emphasize that plantlets transplanted without acclimatization process were prone to microbial attack as the sterile environment they grew from did not encourage defense system development against these attacks. It was proven that hardening of the plantlets improve their tolerance towards stress especially moisture (Takatori et al., 1967). Thus, acclimatization or hardening must be considered as a stage in tissue culture plantlets if the full benefits of tissue culture were to be met.

Young plants about 2 to 3 weeks with a height range lower than 5 cm were not able to establish steady disease progression during infection assay due to imperfect vascular development (Mak et al., 2004). Thus, to perform a success bioassay study, host plants

with fully develop root system is required. In our present study, we select 2 month old healthy tissue culture banana plants with minimum 3 to 5 green leaves, vigorous healthy white roots, and sturdy stem with range of diameter of 0.5 to 1.0 cm. All of these criteria was taken into account due to two essential indicators of *Foc* infection, where the disease evaluation will be carried out through appearance of the leaves and the rhizome.

5.4.2 The host plant

In any plant infection bioassay experiment, the selection of the developmental and physiological stages of the plant material to be used is a paramount consideration as disease response and gene expression profiles may vary considerable depending on the maturity state of the cells. Hence we found that for bananas, the cell type to be assayed should enable the mimicry of the natural modes of plant exposure to the pathogen. For *Foc*, this would typically be through a root contact. Young plants about 2 to 3 weeks with height range lower than 5 cm were not able to steady the disease progression during infection assay due to imperfect vascular development.

The usage of fertilizers and supplements for the plant growth should also be standardized to minimize the variables in the plant physiology. The type, frequency and quantity should not be changed between experiments. In our study, Hoagland's solution was used as the nitrogen fertilizer. Hoagland's solutions is an economical, simple and effective fertilizer (Conn et al., 2013). Study by Bever and co-researchers (2013) revealed that Hoagland's solutions able to induce the best growth in terms of plant height, leaf quantity and foliage color.

The plants need to be maintained in a consistent condition (temperature, humidity and light). The use of growth rooms for this bioassay experiment needs to mimic natural

conditions since the sensitivity to fungal infection heavily depends on the temperature during inoculation (Hudec & Muchová, 2010). In addition, bananas are sensitive to temperature changes. It would densely influence the arrangement of the leaves, vitally contributing to the rate of plant development will be affected as well (Salau et al., 2016; Turner, 1985). With this information, we took note that it may contribute to the differences in pathogenicity profiles. We basically controlled the ambient temperature from 28 to 32℃ to mimic natural temperature where *Foc* infects Berangan.

5.4.3 Optimization of inoculation media

Inoculum standardization is an important parameter in such procedures as optimize desired concentration and reproducibility of the spore suspension influenced in the bioassay results. In the present study, we aim to develop a fast and precise method for evaluating the cell-density of *Fusarium* spore suspension in cv. 'Berangan' bioassay experiment. Two inoculation medium, PDB (Difco^{TM}, BD, France) and Armstrong Liquid media were used as the inoculum culture media and their performance were observed. The best performance of fungal growth from the two types of inoculation will be used for further experiment. Results in our studies shows that PDB (Difco^{TM}, BD, France) was the more suitable inoculation media compared to Armstrong Liquid Media.

Lee and co-researchers (2008) proved that *F. oxysporum* produce maximal number of spores production at 8th days of cultivation in PDB and that it was the fitting inoculation media compared to other media. Same results were obtain by Koley and co-researcher (2015) when they found that, PDB was able to produce a high dry weight growth of fungal following Richard's Broth, Sabouraud's Broth and Malt Extract Broth. Coincidentally, they studied about the growth rate of fungus on PDB medium. They had observed the same pattern of growth reduction upon the highest dry weight growth recorded on the 8th

day of cultivation. Similar growth patterns were also described by Srivastava and co-researchers (2011) wherein the growth rate of *F. oxysporum* increased at a slow rate till 48 hours but thereafter increased at an exponential rate until 8th days of cultivation on the PDB (Difco^{TM}, BD, France). They also observed that after 8th days, its rapid growth stabilized and it trended into a stationary phase. This reduction pattern was comparable to our finding. The reduction of fungal growth rate hypothesized by Koley and co-researcher (2015) was that, the fungal underwent nutrient exhaustion of the medium , leading to lysis of the fungal cells and resulting in the lag phase.

5.4.4 Inoculation technique

The root dipping technique was favored as it was simple and reliable. The two month old plantlets are easily handled and lessen damages to the roots. Masheva and Todorova (2013) in their study on strawberry and pepper plants supported the idea of root dipping would expedite direct contact between the roots and the inoculum. They found that the index of infestation was higher in the root dipping technique compared to that recorded in non-infected plant material planted in infected soil. Apart from the root dipping technique, the infection can be infested by foliar spraying, syringe infiltration, wounding and spore suspension drop (Giri et al., 2013).

An essential parameter that should be taken into account is the time period of plant cell exposure to the spore inoculum. All isolates in our study were exposed for 2 hours with the spore inoculum. This period consistently produced the expected disease progression and response in susceptible Berangan cultivars. Bletsos et al. (2002) also applied a similar method to test the susceptibility of their *Greek Melon* cv. *Kokkini* banana, *Tharaki*, *Peplos* and *Amynteou* to *Verticulun dahlia* with *Fusarium oxysporum* f.sp. *melonis* by immersing their roots for 1 hour. Other studies reported that a 30 min of

inoculum contact was enough to result in decrease in plant growth (*Fusarium* infection symptom) (Singh et al., 2010). We recommended 2 hour dipping for banana cultivars. Other usage of external solvents such as wetting agents are avoided as it may affect the actual event in the field (Abouzeid & El-Tarabily, 2010).

5.4.5 Validation of disease progression

The bioassay pathogenicity tests that were performed in our study demonstrated that the *Foc* isolates found in banana tissues in Malaysia are able to re-colonize the banana vascular tissue, primarily in the rhizome and pseudostem region. The rhizome section is the main nutrient reserve or nutrient storage part of the banana plant ("Rhizome," 2018). The fungus would be using this reserve as their nutrient source (Ma et al., 2013). Both race 1 and 4 isolates tested infected the Musa acuminata cv. Berangan with different levels of virulence detected.

The external symptoms observed during bioassay tests included leaf yellowing, wilting and stem base splitting. Our preliminary bioassay experiments was performed using C1HIR isolates, exhibit race 4 and VCG 01213/16 characteristic. We noted that, on both trials, cv. 'Berangan' shows DSI of highly susceptibility for both sets of trials. Once the preliminary experiment has done and all precaution procedure were controlled, the experiment was then performed with other *Foc* isolates. A total of 2 *Foc* isolates race 1 (2306 and 10201) and race 4 (2296 and 9888) was then chosen in random for bioassay experiment. Our results show that the earliest external symptoms for race 1 infection were detected on the 4^{th} week while race 4 was able to exhibit symptoms on the second week.

Cultivar 'Berangan' showed susceptible results towards isolates 2306 exhibits race 1 of *Foc* while isolate 10201 was significantly susceptible. The difference in the virulence

of this race may have a connection with their VCG. However, we cannot conclude this statements, as we do not have complete details of VCG for isolate 10201. Reports for *Foc* race1 infection by most researchers show that external symptoms appeared between the 3rd and 4th week under controlled conditions (Sivamani & Gnanamanickam, 1988; Stover, 1959). Paul et al. (2011) demonstrated that *Foc* race 1 was able to infect the 'Lady Finger' variety as early as 3 to 5 weeks of post-inoculation. Experiments by Sabadel et al. (2003) on the variety 'Chuoi' (AAA) demonstrate that external symptoms developed visibly on the 12th week. A group of researchers in Australia, Paul et al. (2011), performed a bioassay glasshouse trial in order to assess their transgenic banana lines in response towards *Foc* race 1, VCG 0124/5. They used wild type 'Lady finger' as wild-type positive control and they notice the first appearance of typical external disease symptoms within 3 to 5 weeks.

For experiment using *Foc* race 4 isolates (2296 and 9888), our test showed the host to be susceptible to the isolates tested with earliest external symptoms typical observed on the second week as mentioned earlier. These results concurred with our preliminary study using *Foc* race 4 isolates C1HIR. *Foc* isolates 2296, 9888 and C1HIR shared the same VCG of 01213/16, thus the same pattern of pathogenicity were observed as expected.

The use of RDI and LSI was used to determine whether similar or varying infection manner are established across plant material. As of now, there were no single standard RDI and LSI reported. Nasir et al. (2003) reported that disease symptom progression of *Fusarium* beyond score 2 was very rapid. Jie et al. (2009) indicated that wilt severity need to be assessed based on a rating scale from 0 - 4, where 0 is considered with no infection while score 1 is when 1 - 25% infection, score 2 when 26 to 50% infection, score 3 equivalent to 51 to 75% infection and finally score 4 when 76 to 100 of *Foc* infection.

We suggest that each plant variety needs to have its own standardized LSI and RDI while closely related species can use a single protocol without any bias.

5.4.6 Precaution action during bioassay study

Throughout this whole study, we have noted several critical precautions that should be treated as decisive issue to address. During our literature study, very little information was found on precautions during bioassay studies especially during soil preparation (acclimatization stage) and clean-up procedure after bioassay study were performed. Soil and sand used in herein was sterilized and air-dried for at least 3 days inside a greenhouse. This is to make sure the elimination of gases formed during the sterilization process. Pérez-vicente et al. (2014) in his manual had emphasized this concern and had mentioned that they observed phytotoxic effects when using autoclaved soil on banana experiment due to incorrect steps of post-autoclave aeration.

In this study, the double tray approach was applied to perform the bioassay study of *Foc* infection. The irrigation water was placed in the outer tray to avoid contamination on the greenhouse surfaces. The drained water were then treated with sodium hypochlorite before discharged. Once the whole experiment is complete, pots were extensively sterilized together with other substrate and apparatuses used. Pérez-vicente et al. (2014) recommend to autoclave the contaminated soil twice with 24 hour interval upon discharge.

5.5 Differential gene expression in banana roots in response to *Foc*

Identification of defense-related genes in the host is one of the most essential steps in understanding the mechanism of disease resistance in plants (Raman et al., 2016).

Analysis of these genes is a complementary assay to the standardized bioassay described earlier. The role of 3 defense genes evaluated in this study with the RT-qPCR analysis has provided an insight into the defense-related gene expression in bananas mediated through *Foc* interactions. The defense response of a plant against pathogen attack is an aggressively regulated chain of events and may result the onset of resistance or the establishment of the disease (Attard et al., 2010). Upon pathogen recognition, signaling events trigger a series of early cellular responses and physiological changes in the plants such as changes in ion fluxes across the membrane, synthesis of reactive oxygen species (ROS) and also changes in its gene transcription (Wu et al., 2014). Delayed defense response however included the production of antimicrobial compounds, cell wall strengthening, and the triggering of systemic acquired resistance (SAR), which results in a long-lasting resistance that usually establish in non-infected areas of the plant (Romeis, 2001). The genes investigated in this study fall into these two categories and can be further divided into basic functional group. These categories are

1) *PAL* involves the phenylpropanoid pathway and cell wall strengthening and;

2) *PR10* are antifungal proteins.

Liu et al. (2006) suggested that *PR10* RNase activity protect the plants during the programmed cell death around infection sites (acting directly on the pathogens).

The genes investigated in this study have shown that they are potentially key genes in the defense response of the susceptible Berangan towards *Foc* infection. Expression of both genes, *PAL* and *PR10* continued to increase during infection by race 1 of *Foc* on all time points analyzed. Interestingly, infection caused by race 4 resulted in the reduction of expression for both genes tested at 2 dpi. The genes then showed significant upregulation at day 4 of post-inoculation. This observation suggests that the pathogen may invade the plant effectively by suppressing expressions of defense-related genes thus evading detection (Dowd et al., 2004). The expression pattern of genes by different races of *Foc*

in this study show obvious differences which should be studied further. Detailed function and pathway of both genes involved in plant defense response are described below.

5.5.1 Phenylpropanoid pathway

Phenylpropanoid pathway enzymes (Figure 5.1) are important defense-related compounds that includes *PAL* and other crucial compounds. The upregulation of these genes may cause the increase of lignin production which is critical for cell wall strengthening, flavonoids, isoflavonoids and stilbenes upon pathogen attack (Dixon et al., 2002). *PAL* activities during infection by *Foc* race 1 and 4 in our study show different differential expression patterns as mentioned earlier. Continuous expressions was detected on the sample infected by *Foc* race 1. When infected by *Foc* race 4, the gene downregulated on 2 dpi but later upregulated on 4 dpi. This expression pattern is similar to the experiment led by Fan et al. (2017). Their group observed that the *PAL* activity in *Foc* race 4 downregulated from 6 to 12 hours of post inoculation (hpi) but then upregulated on 24 hpi, showing the highest expression on 48 hpi. Paparu et al. (2007) perform a study on gene expressions of *PAL* susceptible in the *Musa acuminata* cv. 'Nabusa' with a non-pathogenic strain of *Fo.* Their results show similar patterns of dramatic expressions. He pointed out that the reason of the drastic decline as observed in 2 dpi of race 4 infection in our studies are unclear, but one can presume that it is necessary for the establishment and development of endophyte-banana symbiosis. Paparu et al. (2007) also emphasized that the later raise in *PAL* activity might contribute directly or indirectly to cell wall strengthening. It is clearly explain that phenylpropanoid pathway products show important roles as signal molecules, both in plant development and especially for plant defense mechanism. The best-known examples of regulatory roles for phenylpropanoid pathway may include the activities of SA as a regulator of both local

and systemic pathogen-induced defense gene activation, the oxidative burst, and pathogen-induced cell death (Nimchuk et al., 2003).

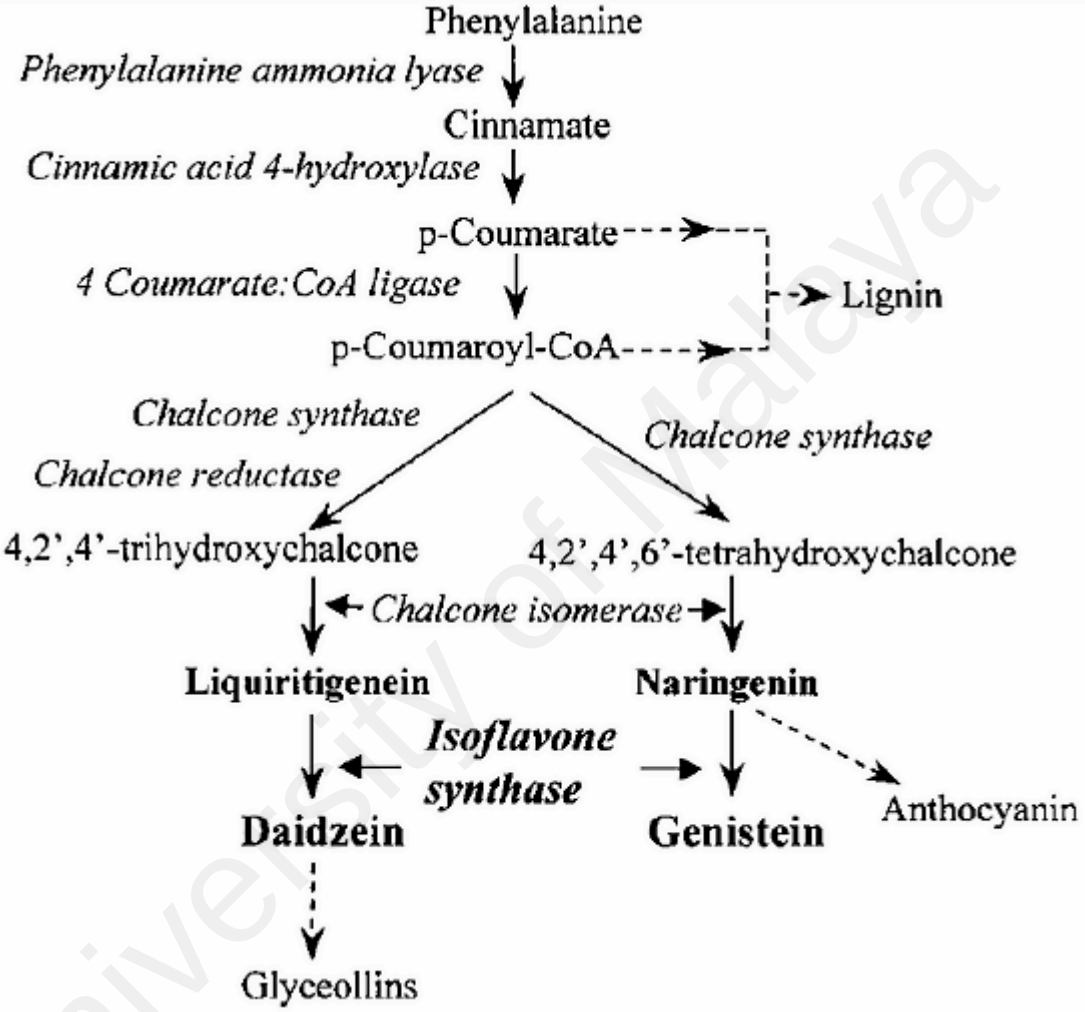


Figure 5.1: A simplified diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis. Citation report graphic is derived from Springer Nature, with permission from Woosuk Jung et al. (2000).

5.5.2 Antifungal protein

Plants have developed a variety of defense mechanisms to protect themselves against pathogen attacks, which includes synthesis of low molecular weight compounds, proteins, and peptides that have antifungal activity. Antifungal protein which are involved in either

constitutive or induced resistance to fungal attack (Selitrennikoff, 2001). PR protein is one of the mentioned antifungal proteins and it has been classified into no less than 17 groups of induced proteins (Table 5.1). The families are numbered in the order they were discovered (Van Loon et al., 2006).

Table 5.1: Recognized families of pathogenesis-related proteins comprised of 17 groups.

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr 1*
PR-2	Tobacco PR-2	B-1,3-glucanase	*Ypr 2, (Gns2 (Glb))*
PR-3	Tobacco P, Q	Chitinase type I,II,IV,V,VI,VII	*Ypr 3, Chia*
PR-4	Tobacco 'R'	Chitinase type I,II	*Ypr 4, Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr 5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr 6, Pis ('Pin')*
PR-7	Tomato P_{69}	Endoproteinase	*Ypr 7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr 8, Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	*Ypr 9, Pr*
PR-10	Parsley 'PR1'	Ribonuclease-like	*Ypr 10*
PR-11	Tobacco 'class V'	Chitinase, type I	*Ypr 11, Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr 12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr 13, Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr 14, Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr 15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr 16*
PR-17	Tobacco PRp27	Unknown	*Ypr 17*

Many gene expression studies were conducted between the host and *Fusarium* species to evaluate the expressions of the *PR*s gene. Our recent investigation studies of the *PR10* gene interactions with *Foc* race 1 and race 4. Our results revealed that *PR10* genes continuously upregulate during the infection by *Foc* race 1. As for *Foc* race 4 infections, the gene downregulates on 2 dpi but then upregulates on 4 dpi. Studies conducted by Godfrey et al. (2012) on PRs protein revealed that continuous incline of expressions were observed during the infection by *Foc* race 1 but the expressions downregulated after 51 hours of infection by *Foc* TR4. The results of both our study groups are corresponding to each other. Extensive study on *Fusarium* species towards their host were done. Experiment performed by Xing et al. (2016) on the interactions between *Fusarium oxysporum* f. sp. *conglutinans* in cabbage revealed that *PR10* was induced after inoculation. Other studies on the interaction between *Fusarium oxysporum* f. sp. *vasinfectum* in cotton root also revealed that the *PR10* constitutively expressed in roots on every time point tested, 3 and 4 dpi, conducted by Dowd et al. (2004). They also emphasized that the function of *PR10* is still unclear but they are evidently involved in the response mechanism of the host towards pathogen attacks.

CHAPTER 6: CONCLUSION

The results obtained in this study demonstrated that the use of molecular methods establishes an important complement to the morphological criteria needed to allow fungi to be more easily identified.

The pathogenicity tests demonstrated that all *Foc* isolates recovered from diseased banana tissue in Malaysia were able to recover on PDA media and had significant levels of virulence differences. Difference of virulence level was observed from different race and VCG that they belong. In this study, inoculation of cv. 'Berangan' with $10x^6$ spores/ml of fungal suspension for two hours using roots dipping technique and 'double-tray' set-up have been selected as the bioassay parameter. External wilting symptom on the leaves can be visualized as early in the 1st week of post-inoculation. More severe infection by *Foc* was exhibit by plants' death. Internal discoloration in the rhizome region of infected cv. 'Berangan' demonstrated brownish discoloration which may extent to the pseudostem. Wild 'Berangan' plants were found to be highly susceptible to all *Foc* race 4 and *Foc* race 1 isolate 10201 tested. However, the wild 'Berangan' were found to be susceptible to *Foc* race 1 isolate 2306. The validated inoculation conditions can be used as references for future bioassay experiments using other *Foc* isolates and banana cultivars. In this study, a systematic bioassay procedure for testing *Fusarium* infection in cv. 'Berangan' banana has been established. This standardized assay facilitates cross confirmation in quality control and assurance that can be carried out by independent laboratories. By considering both the phenotypic symptoms as well as the gene expression profiles in determining the overall disease progression, the integrity of the findings will be strengthened against false positive as well as false negative and this is especially vital for regulatory compliance in risk assessment procedures for transgenic plants.

In conclusion for gene expression analysis, this study established that, shortly after infection, there is differential expressions of defense-related genes among susceptible *Musa acuminata* cv. 'Berangan' inoculated with *Fusarium oxysporum* f. sp. *cubense*. This phenomenon support the notion that early recognition is crucial for resistance cultivar. Detection of early induced defense-related genes are useful in breeding programs for the selection of resistance cultivar of banana against Fusarium wilt. Furthermore, we conclude that, detection of over-expression of *PR10* and *PAL* are indicative of defense responses in the banana-*Foc* interaction. The information gained from this study would benefit future genetic improvement of banana, particularly in *Fusarium*-banana interaction studies.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS:

Baharum, N. A., Othman, R. Y., Mohd-Yusuf, Y., Tan, B. C., **Zaidi, K.,** & Khalid, N. (2018). The effect of *Pathogenesis-Related 10 (Pr-10)* gene on the progression of Fusarium wilt in *Musa acuminata* cv. 'Berangan'. *Sains Malaysiana, 47*(10), 2291-2300.

Munusamy, U., Yusuf, Y. M., Baharum, N. A., **Zaidi, K.,** & Othman, R. Y. (2019). RT-qPCR profiling of pathogenesis related genes in *Musa acuminata* cv. 'Berangan' seedlings challenged with *Fusarium oxysporum* f. sp. *cubense* tropical race 4. *Pakistan Journal of Agriculture Science, 56*(1), 37-42.

Munusamy, U., Mohd-Yusuf, Y., **Zaidi, K.,** Baharum, N. A., & Othman, R. Y. (2019). Evaluation of pathogenicity level of three different strains of *Fusarium oxysporum* in *Musa acuminata* cv. 'Berangan'. *Research Journal of Biotechnology, 14*(3), 12-20.

PAPER PRESENTED:

Zaidi, K., Yusuf, Y. M., Baharum, N. A., Munusamy, U., & Othman, R. Y. (2015). *Development of standardized fungal susceptibility testing methods for Fusarium oxysporum f. sp. cubense challenged experiment in cv. 'Berangan' banana.* Poster presented at the 22nd Society for Molecular Biology and Biotechnology (MSMBB) Scientific Meeting, Kuala Lumpur, Malaysia.