CHARACTERIZATION OF *Secreted in Xylem* (*SIX*) GENES DURING PLANT- PATHOGEN INTERACTION OF *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 IN *Musa acuminata* **cv. 'Berangan'**

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FACULTY OF SCIENCE
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KAUSALYAA A/P KALIAPAN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **KAUSALYAA A/P KALIAPAN**

Matric No : **17088646/2**

Name of Degree : **MASTER OF SCIENCE**

Title of Dissertation ("this Work"):

CHARACTERIZATION OF *Secreted in Xylem* (*SIX*) GENES DURING PLANT PATHOGEN INTERACTION OF *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 IN *Musa acuminata* cv. 'Berangan'

Field of Study : **LIFE SCIENCES (PLANT BIOTECHNOLOGY)**

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CHARACTERIZATION OF *Secreted in Xylem* (*SIX*) GENES DURING PLANT-PATHOGEN INTERACTION OF *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 IN *Musa acuminata* cv. 'Berangan'

ABSTRACT

Fusarium wilt of banana is a lethal disease caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), a soil-borne pathogen that curbs the production of this crop. Small effector proteins known as Secreted in Xylem (SIX), are secreted into the xylem sap of the host plants triggering virulence and interferes with the defence responses of the host plants. Knowledge and comprehension of the interaction between *Foc* and banana plants during the infection is essential to expand methods to mitigate the disease's damage. Therefore, studies on *SIX* genes are crucial as they have significant impact on *Foc*'s pathogenicity and play substantial role in the development of molecular framework and disease control approaches for banana. In this study, the responsible *SIX* genes for this pathogenic reaction were identified, characterized and their gene expression patterns were analysed during the infection. A comparative whole genome analysis was also carried out between Malaysian *Foc*1_C2HIR and *Foc*4_C1HIR isolates. A total of 48 cv. 'Berangan' plantlets were used where 32 plantlets were subjected to *Foc* infection (*Foc*1 and *Foc*4) while 16 plantlets were used as controls (without infection). Collection of plant samples were made at four different time points, 0 day, 2 weeks, 4 weeks and 5 weeks for molecular studies while the internal and external symptoms were confirmed by visual assessment and scored based on the percentage of infection on the 5th week post-inoculation. Following the data analysis, the 'Berangan' cultivar was confirmed to be highly susceptible to *Foc* infection. The Rhizome Discoloration Index (RDI) and Leaf Symptom Index (LSI) of *Foc*4-inoculated plants (7.25; 4.25 respectively) were higher compared to *Foc*1-

inoculated plants (5.0; 3.25 respectively) which suggested that *Foc*4 is more virulent than *Foc*1 in cv. Berangan. Seven *SIX* genes i.e. *SIX1, SIX2, SIX4, SIX6, SIX8a, SIX9a, SIX13* were identified to be present in *Foc*4 DNA, while only *SIX9b* was identified in *Foc*1 DNA and validated via sequencing analysis. Five of the *SIX* genes were shortlisted to be further studied on their expression pattern during infection by performing real-time PCR (qPCR) on fungal RNA from infected banana root samples. *TEF1α* and *TUB* were selected as the endogenous genes for normalisation. In the gene expression analysis conducted on *Foc*1 and *Foc*4 infected plants, two different patterns of expression were observed. In *Foc*4-inoculated plants, *SIX1, SIX6, SIX8a* and *SIX9a* were significantly upregulated on the 2nd and 5th week, whereas in *Foc*1-inoculated plants, significant upregulation of *SIX9b* was observed at a later stage which was on the 5th week. Besides, whole genome sequencing analysis for both *Foc*1_C2HIR and *Foc*4_C1HIR Malaysian isolates revealed that they had a respective genome size of 53 Mb and 55 Mb. Further analysis also revealed the genome features, *SIX* gene structures, putative virulence-associated genes, and functional groups of proteins identified in C2HIR and C1HIR isolates. These findings will aid in understanding the mechanism of potential disease control targets in these plants, including the improvement of diagnostics and breeding programmes.

Keywords: Banana, Fusarium wilt, *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Secreted in Xylem* (*SIX*) gene expression, Whole Genome Sequencing

CHARACTERIZATION OF *Secreted in Xylem* (*SIX*) GENES DURING PLANT-PATHOGEN INTERACTION OF *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 IN *Musa acuminata* cv. 'Berangan'

ABSTRAK

Penyakit kelayuan Fusarium adalah penyakit berbahaya yang memusnahkan tanaman dan disebabkan oleh *Fusarium oxysporum* f.sp. *cubense* (*Foc*), patogen bawaan tanah yang menjejaskan pengeluaran tanaman pisang. Protein efektor yang dikenali sebagai 'Secreted in Xylem' (SIX), dirembeskan ke dalam sap xilem tumbuhan perumah yang mencetuskan virulensi dan mengganggu gerak balas pertahanan tumbuhan perumah. Pengetahuan dan pemahaman tentang interaksi antara *Foc* dan jangkitan pada tanaman pisang adalah penting untuk mengembangkan kaedah untuk mengurangkan kerosakan yang disebabkan oleh penyakit ini. Oleh itu, kajian ke atas gen *SIX* adalah penting kerana ia mempunyai kesan yang signifikan terhadap sifat patogenik *Foc* dan memainkan peranan yang penting dalam pembangunan rangka kerja molekul dan pendekatan kawalan penyakit untuk tanaman pisang. Dalam kajian ini, gen *SIX* yang bertanggungjawab untuk tindak balas patogen ini telah dikenalpasti, dicirikan dan corak ekspresi gen tersebut telah dianalisis semasa jangkitan. Sebuah analisis lengkap penjujukan genom juga telah dilakukan antara isolat *Foc*1_C2HIR dan *Foc*4_C1HIR yang dikenalpasti di Malaysia. Sebanyak 48 kultivar pisang Berangan telah digunakan dan sejumlah 32 anak pokok telah dijangkitkan dengan *Foc* (*Foc*1 dan *Foc*4) manakala 16 anak pokok telah digunakan sebagai kawalan (tanpa infeksi). Pengumpulan sampel tumbuhan selepas jangkitan telah dibuat pada empat titik masa yang berbeza iaitu 0 hari, 2 minggu, 4 minggu dan 5 minggu untuk kajian molekular manakala simptom dalaman dan luaran telah dinilai secara visual dan diberi skor berdasarkan peratusan jangkitan pada anak-anak pokok 5 minggu selepas

inokulasi. Berdasarkan analisis data, kultivar Berangan disahkan rentan kepada jangkitan *Foc*. Manakala, 'Rhizome Discoloration Index' (RDI) dan 'Leaf Symptom Index' (LSI) bagi tumbuhan berinokulasi *Foc*4 adalah lebih tinggi daripada *Foc*1 dengan pemarkahan 7.25; 4.25 dan 5.0; 3.25 masing-masing. Ini menunjukkan bahawa *Foc*4 lebih virulen daripada *Foc*1 untuk cv. Berangan. Untuk kajian gen *SIX,* terdapat tujuh gen *SIX* iaitu *SIX1, SIX2, SIX4, SIX6, SIX8a, SIX9a, SIX13* yang telah dikenalpasti dalam DNA *Foc*4 manakala hanya *SIX9b* telah dikenalpasti dalam DNA *Foc*1 dan disahkan melalui analisis penjujukan. Lima gen *SIX* telah disenarai pendek untuk dikaji lebih lanjut mengenai corak ekspresi mereka semasa infeksi dengan melakukan 'real-time PCR' (qPCR) pada RNA patogen *Foc* daripada sampel akar yang telah dijangkitkan. *TEF1α* dan *TUB* telah dipilih sebagai gen endogen untuk normalisasi. Dalam kajian ekpresi gen yang dijalankan pada tumbuhan yang dijangkiti *Foc*1 dan *Foc*4, dua corak ekspresi berbeza diperhatikan. Dalam tumbuhan yang diinokulasi *Foc*4, kesemua *SIX1, SIX6, SIX8a, SIX9a* menunjukkan peningkatan regulasi yang ketara pada minggu kedua dan kelima, manakala dalam tumbuhan yang diinokulasi *Foc*1, gen *SIX9b* hanya menunjukkan peningkatan regulasi ketara pada peringkat yang lebih lambat iaitu minggu kelima. Di samping itu, kedua-dua isolat Malaysia, *Foc*1_C2HIR dan *Foc*4_C1HIR yang telah menjalani analisa lengkap penjujukan genom menunjukkan saiz genom sebanyak 53Mb dan 55Mb masing-masing. Analisa lanjut telah mendedahkan ciri-ciri genom, struktur gen *SIX*, gen yang berkaitan dengan kevirulenan, serta kumpulan protein berfungsi yang dikenalpasti dalam isolat C2HIR and C1HIR. Penemuan ini akan membantu dalam memahami mekanisme sasaran kawalan penyakit yang berpotensi dalam tumbuhan ini, termasuk penambahbaikan program diagnostik dan pembiakan.

Kata kunci: Pisang, penyakit kelayuan Fusarium, *Fusarium oxysporum* f.sp. *cubense* (*Foc*), Ekspresi gen *Secreted in Xylem* (*SIX*), Penjujukan genom lengkap

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'If you have a heartbeat, there's still time for your dreams'

Dreams! That is what drives people to pursue all they have ever desired. My dream has always been to pursue a PhD, and I believe that accomplishing my MSc journey has pushed me one step closer to my aspiration. A tremendously rewarding two years of hard work, sacrifice and beautiful memories to be cherished.

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%	: Percent
±	: Plus/minus symbol
©	: Copyright symbol
®	: Registered trademark symbol
™	: Unregistered trademark symbol
ºC	: Degree Celsius
α	: Alpha
β	: Beta
Σ	: Sum
g	: Standard gravity (relative centrifugal force)
g	: Gram
M	: Molar
ml	: Millilitre
mM	: Millimolar
ng	: Nanogram
nm	: Nanometer
V	: Volt
v/v	: Volume over volume
w/v	: Weight over volume
µg	: Microgram
µl	: Microlitre

A260/230 : Ratio of absorbance at wavelength 260 nm and 230 nm

A260/280 : Ratio of absorbance at wavelength 260 nm and 280 nm

BLASTn : Nucleotide-nucleotide BLAST

BLASTp : Protein-protein BLAST

bp : Base pairs

BUSCO : Benchmarking Universal Single-Copy Orthologs

cDNA : Complementary deoxyribonucleic acid

CDS : Coding sequence

COG : Cluster of Orthologous Group of proteins

Ct : Cycle threshold

cv. : Cultivar

DNA : Deoxyribonucleic acid

dpi : Days post inoculation

DSI : Disease Severity Index

EDTA : Ethylenediaminetetraacetic acid

f.sp. : Formae speciales

Fo : *Fusarium oxysporum*

Foc : *Fusarium oxysporum* f.sp. *cubense*

*Foc*1 : *Fusarium oxysporum* f.sp. *cubense* Race 1

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

gDNA : Genomic DNA

ITS : Internal transcribed spacer

kb : Kilobase

LSI : Leaf Symptom Index

Mb : Megabases

MEGA : Molecular Evolutionary Genetics Analysis

min : Minute

mRNA : Messenger RNA

NaCl : Sodium chloride

NaOAc : Sodium acetate

NCBI : National Centre for Biotechnology Information

PCR : Polymerase Chain Reaction

PDA : Potato Dextrose Agar

PDB : Potato Dextrose Broth

PHI : Pathogen Host Interaction

qPCR : Quantitative Polymerase Chain Reaction

RDI : Rhizome discoloration index

RNA : Ribonucleic acid

RNase : Ribonuclease

rpm : Rotation per minute

S : Svedberg

sec : Second

SIX : Secreted in Xylem

sp. : Species

spp. : Species (plural)

TBE : Tris-boric acid ethylenediaminetetraacetic acid

TR4 : Tropical Race 4

VAG : Virulence associated genes

VCG : Vegetative compatibility groups

wpi : Weeks post-inoculation

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CHAPTER 1: INTRODUCTION

1.1 Background

The banana which includes plantain is an essential fruit crop globally, with about 155 million tonnes of annual production (http://faostat.fao.org, 2020). It is perhaps one of Malaysia's major fruit crops, grown for both domestic and international markets. However, this crop's production has unfortunately been curbed by numerous diseases despite being a prominent player in the agricultural sector globally. Fusarium wilt is a fungal disease that is recognised as highly destructive in the agricultural history, brought about by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) (Li et al., 2012). *Foc* has been classified into four races but only three of them infect banana cultivars, i.e., race 1, race 2, and race 4 (Cheng et al., 2019). However, our current study focuses on race 1 (C2HIR) and race 4 (C1HIR) Malaysian isolates since these are the virulent isolates that frequently infect the banana cultivars grown in our country.

Foc infects banana roots obstructing vascular tissues, and subsequently hindering the dispersion of fundamental minerals to other regions of the plant. The development of Fusarium wilt disease spread had a negative influence on yield and caused production constraints in a few primary banana-producing and exporting localities, including Malaysia (Ahmad et al., 2020). Therefore, understanding the molecular basis of virulence in the pathogen is crucial to carry out further research in combating this disease.

During host infection, one of the effector proteins secreted by plant pathogens (*Fusarium oxysporum*) is Secreted in Xylem (SIX). These effector proteins are necessary for infection because they influence the host's immune response (Czislowski et al., 2021). A study on pathogen-induced genes may prompt a superior comprehension of the molecular processes associated with resistance and may add to the advancement of biotechnological approaches to fight the disease.

These recent advancements in comprehending *Fusarium oxysporum* pathogenicity related to effector proteins have been created after the discovery of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*)'s genome, that taints tomatoes. This prompted the revelation of lineage-specific mobile pathogenicity chromosomes which carry genes related to pathogenicity such as *Secreted in Xylem* (*SIX*) genes that produce tiny effector proteins discharged by *Fol* during tomato plant colonization (Taylor et al., 2016). According to Poon et al. (2019), *SIX* genes' homologues were found in *Foc*TR4 genome and differentially expressed *SIX* genes were found in *Foc*TR4-infected banana roots. Likewise, Czislowski et al. (2021) stated that different races of *Foc* were associated with specific *SIX* genes' homologues. These findings substantiate the hypothesis of the proposed research that different sets of *SIX* genes are expressed in *Foc*1_C2HIR and *Foc*4_C1HIR Malaysian isolates during plant-pathogen interaction.

The intent behind this study is to identify the responsible *SIX* genes and their expression during infection by Malaysian *Foc*1_C2HIR and *Foc*4_C1HIR isolates in cv. 'Berangan' plants. It is noteworthy that, the decision to study the expression of *SIX* genes in cv. 'Berangan' plants in this study will assist in the identification of potential biomarker for early detection of the disease during Fusarium wilt infection in banana as well as supports the future studies in developing a strategic molecular framework to repress the plant-pathogen interaction from causing this lethal infection. Additionally, understanding and analysing the fungal genome from different races (Race 1 and Race 4, that has different pathogenicity level) will reveal the genomic features and aid in elucidating the organization of pathogenicity-associated genes. This will provide a better comprehension on the genomes of the Malaysian isolates and help in development of race-specific control measures. Hence, the yield and production of banana plants can be increased.

1.2 Problem statement

The efficiency of banana and plantain-based farms has been declining due to a fungal disease caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*). Early detection is required to prevent the spread of this disease at its earliest stage as delayed detection would make eradication challenging because the fungus has high fungicide resistance, and their spores can remain dormant in the soil for up to 30 years. Therefore, identifying the responsible virulence gene that can serve as a potential biomarker is essential to detect this disease before any symptom appearance so that proper strategies can be implemented for disease control measures and pest management to curb the disease from developing and spreading further. Additionally, there is a lack of information on *Foc*1 and *Foc*4 Malaysian isolates. For better understanding on the virulence mechanisms of the pathogen, accurate genomic information is vital. Hence, our research project focuses on identifying the responsible *SIX* genes and their expression pattern during *Foc*-banana interaction. Furthermore, the comparative analysis on *Foc*1 and *Foc*4 genome can assist in identifying the variations between these genomes specifically on the virulence-associated gene studies. Taken together, these findings may contribute to the development of early detection framework.

1.3 Objectives

a) To identify the *SIX* genes that are responsible for pathogenic reaction during *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 infection in *Musa acuminata* cv. 'Berangan'.

b) To analyse the expression of selected *SIX* genes during plant-pathogen interaction of *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 infection in *Musa acuminata* cv. 'Berangan'.

c) To perform comparative analysis between *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 genomes of Malaysian isolates.

1.4 Research questions

a) Which *SIX* genes are responsible for pathogenic reaction during *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 infection in cv. 'Berangan'?

b) Which *SIX* gene(s), has/have high expression during the interaction of *Fusarium oxysporum* f.sp. *cubense* (*Foc*), *Foc*1 and *Foc*4 in cv. 'Berangan'?

c) What are the differences between *Foc*1 and *Foc*4 genomes of Malaysian isolates?

1.5 Research hypothesis

Different sets of *Secreted in Xylem* (*SIX*) genes are expressed in *Foc*1_C2HIR and *Foc*4_C1HIR Malaysian isolates during plant-pathogen interaction.

1.6 Significance of the study

This study will provide a better comprehension on the relevance of *SIX* genes during plant-pathogen interactions in *Musa acuminata* cv. 'Berangan'. The identification of responsible *SIX* genes during pathogenic infection will aid in early disease detection as it has a potential to serve as a biomarker. As a result, it will assist in developing a molecular framework and disease control approaches in banana once this pathogen interaction is understood. Additionally, comparative analysis on the *Foc*1_C2HIR and *Foc*4_C1HIR genomes will reveal the details on the genome structure of these pathogens which will enhance the comprehension on *Foc* genomes of Malaysian isolates, hence helps in the advancement of efficient strategies for managing Fusarium wilt disease. These findings will eventually aid in expanding the yield and production of banana plants in the future as well as improve food security since bananas constitute a major staple food crop globally.

CHAPTER 2: LITERATURE REVIEW

2.1 Banana

2.1.1 General background

The banana which includes plantain is an essential fruit crop globally, with about 155 million tonnes of annual production (http://faostat.fao.org, 2020). It is perhaps one of Malaysia's most important fruit crops, grown for both domestic and international markets. Bananas are the world's fourth most significant agricultural product behind rice, wheat, and corn, with over 100 billion bananas consumed worldwide and over 330,000 metric tonnes of banana output providing export earnings of roughly USD 8 million (Ahmad et al., 2020). *Musa* spp., the edible banana is one of the domesticated crops that originated in Asia (Mostert et al., 2017). Most of the banana that are edible belong to the Eumusa section (East Asian genus with 22 chromosomes) and are diploid or triploid hybrids from *Musa acuminata* (A-genome) or hybridisation with *Musa balbisiana* (B-genome). The majority of the cultivars are natural hybrids (Ploetz, 2015) and normally, they are vegetatively propagated, parthenocarpic, triploid and sterile (Forsyth et al., 2006; Perrier et al., 2011). Although there are many cultivars that exist, yet only two of the 50 banana subgroups largely produce this fruit, with the subgroup of Cavendish accounting for beyond 40% of the global total (Ploetz, 2015). However, based on research by Khazanah Research Institute, 'Berangan' is one of the most cultivated and commercialized banana cultivars in Malaysia, grown in the banana producing regions of Johor, Pahang and Sarawak (https://www.krinstitute.org). This statement is supported by statistics from the Department of Agriculture Sarawak, indicating that approximately 50% of the banana plantations in Malaysia are cultivated with Berangan and Cavendish varieties (https://doa.sarawak.gov.my).

2.1.2 Challenges in banana production

Banana yield and productivity, like those of other plants, are influenced by numerous biotic and abiotic stress factors that occur in the banana plants' natural vicinity. As such, banana plants are sensitive to low temperatures, salinity and drought in abiotic stress, resulting in severe losses (Ghag and Ganapathi, 2017). Biotic stress is primarily caused by a variety of diseases and pests, which will inhibit the production of banana plantations if they exceed a particular threshold level in an area. The development of banana industry is directly impacted by the diseases that have struck and continue to afflict this significant resource in global commerce. Diseases are the primary hazard to both commercial banana production for international market and considerably bigger subsistence production for local use. Bacteria, fungi, insects and plant parasitic nematodes wreak havoc on banana yields (Holscher et al., 2014). Some of the most destructive banana diseases include Black Sigatoka (leaf spot disease), Moko disease, Fusarium wilt, fruit rots as well as viral infections like banana bunchy top virus and banana mosaic virus (Bakry et al., 2009; Ghag and Ganapathi, 2017). These diseases can rapidly spread to cause epidemics, owning to monoculture practices, and can cause scarcity in large banana-growing regions (Ghag and Ganapathi, 2017). One of the key threats that has garnered greater attention and considered as the most crucial disease affecting bananas globally is Fusarium wilt, produced by a type of fungus known as *Fusarium oxysporum* f.sp. *cubense* (*Foc*) (Blomme et al., 2017).

2.2 Fusarium wilt disease

Fusarium wilt is a common infection in a wide range of soil worldwide. It is a fungus-induced soil-borne disease brought about by *Fusarium oxysporum* (*Fo*) that is considered as a major vascular disease on crops (Swarupa et al., 2014). In an international community's study, *Fo* was placed fifth in the list of top ten fungal plant pathogens (Dean at al., 2012). *Fo* isolates can survive and develop in soil as well as the rhizosphere of numerous plants for an extended period (Fravel et al., 2003). Pathogenic isolates of *Fo* can cause wilt disease by penetrating the roots (Fourie et al., 2011). Some are non-pathogenic isolates that can taint and colonise the roots but does not cause wilt disease by entering the xylem tissue (Sutherland et al., 2013; Ghag et al., 2015). The spores can remain dormant in the soil lasting up to 30 years, making the disease control challenging. The disease can spread from one region to another in a variety of ways, including through the transfer of contaminated seeds and planting materials as well as machinery and farm tools particularly after they are utilised in soils that are contaminated and reused in healthy fields (Joshi, 2018).

2.3 The genome of *Fusarium oxysporum*

The pathogens' complete genetic and physical maps is an excellent way to explore the content and genome size variation within the genus (Ma et al., 2010). A two-speed genome organization was revealed through genome wide analysis of *Fusarium oxysporum.* The genome was discovered to be structurally and functionally divided into two parts; the core genome which encodes housekeeping genes crucial for pathogen's normal development and survival, and an accessory genome which encodes pathogenicity or virulence-associated genes (Croll and McDonald, 2012; Raffaele and Kamoun, 2012; Schimdt et al, 2013; Dong et al., 2015; Fokkens et al., 2018). As of now, *Fol*4287 remains

as the genome that has been most extensively studied and mapped completely into chromosomal sequences. Hence, *Fol*4287 serves as a crucial reference isolate for subsequent studies. *Fol*4287's genome assembly resulted in the mapping of 15 chromosomes, of which 11 were core chromosomes and four were accessory chromosomes (Jangir et al., 2021).

Based on Ma et al. (2010), the accessory genome, which is also alternatively known as lineage-specific (LS) region, supernumerary (SP) or conditionally dispensable (CD) chromosome comprises 19Mb region of genome that contains the chromosomes 3,6,14,15, and scaffolds 27 of core chromosome 1, as well as scaffold 31 of core chromosome 2. *Fol*4287 genome's LS region, which is home to 74% of the genome's transposable elements (TEs) and 95% of its DNA transposons, may be particularly linked to pathogenic adaptability. Genes found in the pathogenicity-associated genomic regions control *Fo*'s host range and specificity (Rep and Kistler, 2010; Williams et al., 2016). Effector proteins, secreted enzymes, transcription factors (TFs) and proteins involved in signal transduction and secondary metabolism are all encoded by these genes (Jangir et al., 2021). When effector proteins interact with their corresponding resistance (R) genes, they either cause a compatible (virulence) response or an incompatible (avirulence) reaction (Jones and Dangl, 2006).

According to Jangir et al. (2021), observed variation in the genome size in the genus has been attributed by activity of TEs, horizontal chromosomal transfer (HCT) and deletion or fusion of genomic regions. The TEs' activity can result in complex genetic material configurations, deletions, and translocations. Based on Ma et al. (2010), the rapid emergence of novel pathogenic lines with a more extensive host range was caused by horizontal transfer of host-specific genes to otherwise genetically distinct lineages. Moreover, horizontal transfer of genes and chromosomes associated with pathogenicity from a pathogenic isolate to a non-pathogenic isolate (Figure 2.1) result in the emergence

of a new virulent lineage (Jangir et al., 2021). Hence, it was hypothesized that the *Foc* lineages' polyphyletic relationship was due to horizontal transfer of effector and pathogenicity genes, including the *SIX* genes (Czislowski et al., 2018).

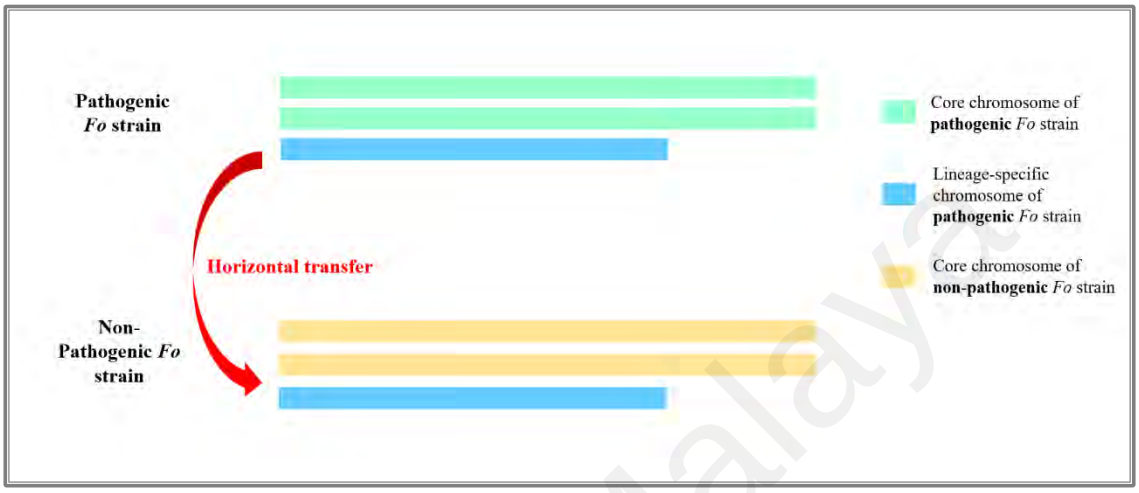


Figure 2.1: Horizontal transfer of lineage-specific chromosome of pathogenic *Fo* isolate to a non-pathogenic *Fo* isolate (Photo modified from Jangir et al., 2021).

2.4 *Fusarium* species

2.4.1 Morphological characteristics

The morphological taxonomy of *Fusarium* species relies largely on the structure and abundance of asexual reproductive structures. These can be observed as chlamydospores, phialides, microconidia and macroconidia as well as the cultural characteristic such as colony structure, colour and cultural aroma (Fourie et al., 2011). *Fusarium oxysporum* is primarily characterized by the presence of non-septate microconidia formed in false heads on short monophialides. Additionally, it exhibits 3-septate macroconidia formed from monophiliades on branched conidiophores in sporodochia. The fungus also produces chlamydospores, which may have a smooth or rough wall appearance, and these chlamydospores are developed either singly or in pairs. This is depicted in Figure 2.2 below. However, according to Nelson, (1991), the identification of *Fusarium* species, can

pose challenges. This difficulty arises from the variability observed between isolates, reflecting a combination of genetic factors and environmental influences impacting the phenotypic expression of morphological features.

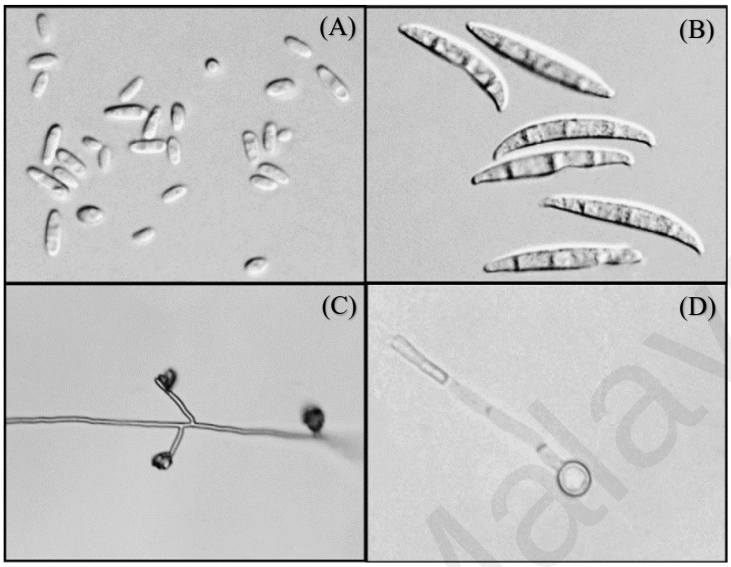


Figure 2.2: Morphological characteristics of *Fusarium oxysporum*. (A) Oval to kidney-shaped microconidia; (B) sickle shaped, thin walled and delicate macrocinidia; (C) microconidia produced in false heads on short monophialides; (D) a single, terminal chlamydospore (Photo sourced from Fourie et al., 2011).

2.4.2 Host range of *Fusarium* species

Fusarium oxysporum (*Fo*) has a diverse host range that includes plantation crops (banana and palms), field crops (cotton and chickpea), flowers (tulips and carnations), and vegetables (bottle gourd and tomato). Despite a wide host range, *Fo* isolates are highly host specific, and morphologically and genetically different. These host-specific forms establish a consortium known as the *Fusarium oxysporum* species complex (FOSC) (Jangir et al., 2021). FOSC is made up of agents that cause vascular wilt, stem-, root-, and crown-rot diseases in economically valuable crops all over the world.

Fusarium wilt isolates are categorized into over 120 formae speciales and races (Raza et al., 2016). They are adapted to infect specific host plants (Jenkins et al., 2021). Most of the formae speciales are pathogenic to a specific crop and isolates that cause infection

on the same crop are classified together (Fourie et al., 2011). To designate the host of the pathogens, *Fusarium oxysporum* naming arrangement is generally followed by forma specialis (f.sp.). Table 2.1 shows few types of pathogens and their host plants.

Table 2.1: Pathogens and their host plants

Pathogens	Host plants	Reference
Fusarium oxysporum f.sp. *cubense*	Banana	Ploetz, (2006)
Fusarium oxysporum f.sp. *lycopersici*	Tomato	Srinivas et al. (2019)
Fusarium oxysporum f.sp. *niveum*	Watermelon	Hao et al. (2010)
Fusarium oxysporum f.sp. *lactucae*	Lettuce	Malbran et al. (2014)
Fusarium oxysporum f.sp. *phaseoli*	Beans	Pereira et al. (2013)
Fusarium oxysporum f.sp. *cucumerinum*	Cucumber	Abro et al. (2019)
Fusarium oxysporum f.sp. *cepae*	Onion	Taylor et al. (2016)
Fusarium oxysporum f.sp. *passiflorae*	Passion fruit	Melo et al. (2020)
Fusarium oxysporum f.sp. *elaeidis*	Palm oil	Rusli et al. (2017)
Fusarium oxysporum f.sp. *dianthi*	Carnation	Ajit et al. (2006)
Fusarium oxysporum f.sp. *vasinfectum*	Cotton	Halpern et al. (2018)
Fusarium oxysporum f.sp. *cannabis*	Hemp	Pellegrini et al. (2021)

Forma specialis is additionally classified into pathogenic races based on virulence towards cultivars of the same host (Correll, 1991). Generally, the emergence of new races within a forma specialis is due to pathogenicity-associated gene mutations (Jangir et al., 2021). This is observed in *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) where, race 1 isolates express three effector genes (*AVR1*, *AVR2*, and *AVR3*). The emergence of race 2 was a consequence of *AVR1* deletion and emergence of race 3 was due to point mutation(s) in *AVR2* (Takken and Rep, 2010; Biju et al., 2017).

2.5 *Fusarium oxysporum* f.sp. *cubense* (*Foc*)

2.5.1 General background

Fusarium oxysporum f.sp. *cubense* (*Foc*), the causative agent of Fusarium wilt in bananas, is one of more than 150 plant pathogenic forms of *Fusarium oxysporum* (Fourie et al., 2011). It has been recognised as a prominent constraint to the production of banana, resulting in enormous economic losses around the world (Siamak and Zheng, 2018; Segura-Mena et al., 2021). *Foc* is a haploid asexual pathogen (Ordonez et al., 2015), and considered as a pathogen with high degree of variability although there are no reports regarding its sexual recombination (Bentley et al., 1998; O'Donnell et al., 1998).

2.5.2 *Foc* physiological races and vegetative compatibility groups

The *Foc* pathogen was classified into different races based on its potential to induce disease in specific host cultivars (Costa et al., 2015). *Fusarium oxysporum* f.sp. *cubense* is classified into four physiological races but only three of them infect banana cultivars; race 1, race 2, and race 4 (Cheng et al., 2019). Race 1 infects the banana cultivars 'Gros Michel' (*Musa* sp. AAA group), 'Silk', 'Pome', and 'Pisang Awak' (*Musa* sp. AAB group). Race 2 taints cultivar 'Bluggoe' and its closely related cultivars. Race 4, on the other hand, infects almost all cultivars including 'Dwarf Cavendish' (*Musa* sp. AAA group) and also race 1 and race 2 host groups (Guo et al., 2014; Deltour et al., 2017). *Foc* Race 4 is perceived as the most virulent and additionally classified as tropical (TR4) and subtropical (STR4) variants (Ploetz, 2006; Leong et al., 2010). *Foc* TR4 and STR4 interacts significantly with temperature where TR4 can infect banana plants in all environments but STR4 isolates infect plants grown in lower temperatures (Ploetz, 2011; Carvalhais et al., 2019).

Furthermore, at least 24 vegetative compatibility groups (VCGs) of *Foc* have been found globally (Meldrum et al., 2012). According to Ploetz (1994), vegetative compatibility groups (VCGs) are the key evolutionary units within the species for many fungi, particularly those like *Foc* that do not sexually reproduce. VCGs are made up of isolates that can anastomose to produce a stable heterokaryon where genetic materials can be exchanged (van der Does et al., 2008; Jelinski et al., 2017). Therefore, vegetative compatibility promotes gene flow, while incompatibility limits genetic interaction. Hence, isolates from the same VCG have a lot in common biologically, physiologically, and pathologically (Fraser-Smith et al., 2014; Carvalhais et al., 2019).

2.5.3 Infection process by *Foc*

The infection begins with fungus-produced macro and micro conidia invading the roots, followed by infecting the vascular tissues, prompting wilting and the plant's progressive demise (Chung, 2012). These fungi are nutrient-dependent on plants and have developed mechanisms to acquire access to the plants. Scientists have studied and recognised structural morphology change, such as hyphae development, as one of the penetration mechanisms. The fungal pathogen develops hyphae that attach to and penetrate the epidermal cells of both wounded and unwounded roots (Joshi, 2018). They colonise root cells and spread to other areas of the plant via the vascular bundles and upstream water flow, also known as the transpiration stream pull of water in vascular tissues, particularly the xylem. Macro and micro conidia will be produced by the fungus while developing and spreading in the vascular tissues (Joshi, 2018). Mycelia development in the vascular growth causes vessel blockage, which promotes water stress and appearance of wilt symptoms. The interaction speeds up when the plants attempt to respond by creating defence components like gels, tyloses, and parenchyma cell multiplication, that leads to even more vessel blockage (Ortiz et al., 2014; Joshi, 2018).

2.5.4 Fusarium wilt disease symptoms in banana

The starting point of the infection is in feeder roots, and the xylem discoloration which turns reddish-brown is the first internal symptom to be observed. The spreading of vascular discoloration extends towards rhizome, a point to connect stele and cortex, and eventually reaches and incorporates large segments of the pseudo stem. The most seasoned leaves on plants aged four months and above turn yellow or longitudinally split at the base. More younger leaves wilt and decay over time until the canopy is entirely made up of leaves that are dying or dead (Ploetz, 2006). Figure 2.3 and 2.4 shows the disease symptoms and disease cycle of *Fusarium oxysporum*.

Figure 2.3: Disease symptoms of Fusarium wilt of banana caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) Race 4 in Kuala Selangor, Malaysia. (A) The affected plants wilted rapidly; the yellow leaves either stand upright or collapse at the petiole and droop down from the pseudo stem (B) A dark brown discoloration is observed in the cross section of the pseudo stem of Fusarium wilt infected plants.

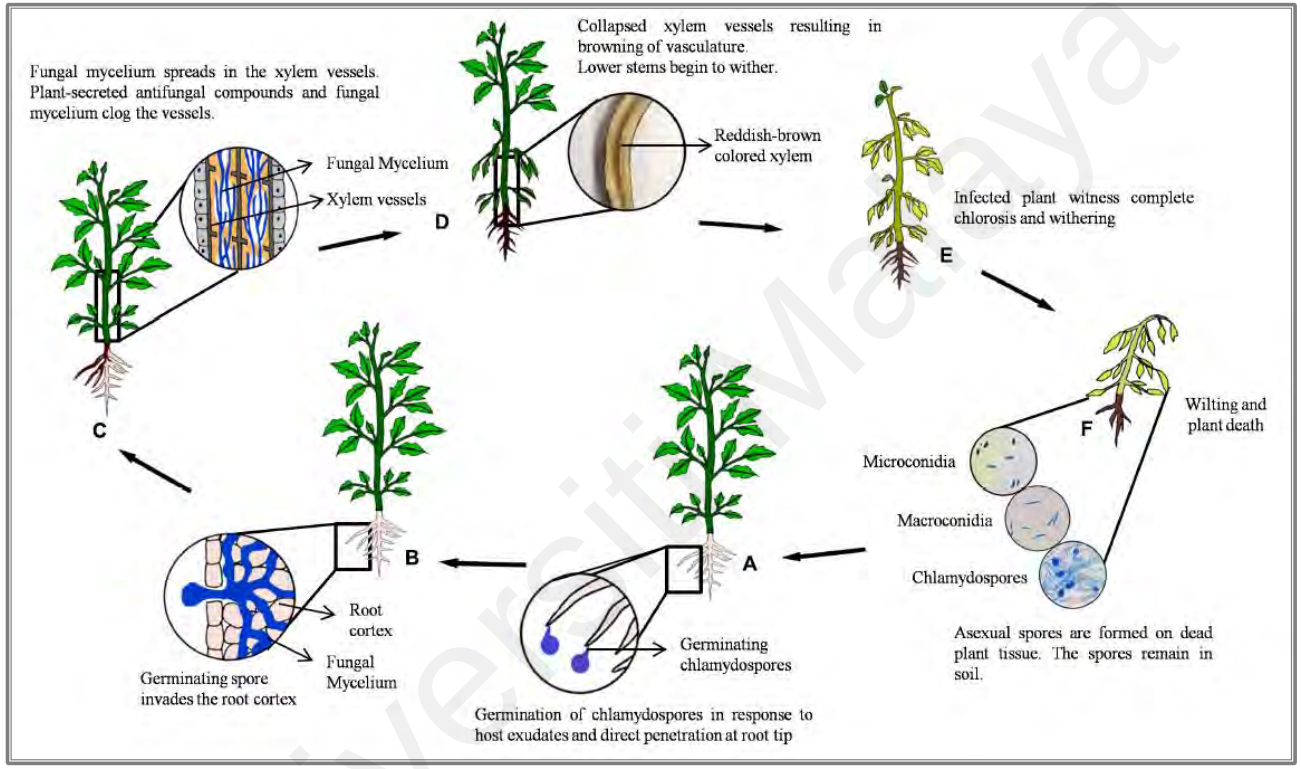


Figure 2.4: Disease cycle of *Fusarium oxysporum*. (A) The exudate secretion of the host plant causes the spores to germinate and the formation of infection hypha, allowing penetration of the root epidermis at the tip. (B) The hypha advances intercellularly through the root cortical cells to xylem tissue, parenchymal cells, and vessels via xylem pits. (C) As a result of excessive mycelial development, the vascular vessels are colonized by the pathogen resulting in browning and blockage (D) The initial infection stage shows symptoms at the stem base and gradually progresses upwards, resulting in the withering of young leaves (E) The mature leaves exhibit minor yellowing or full chlorosis (F) As the disease progresses, the host plant wilts and dies. Fungal spores (microconidia, macroconidia, and chlamydospores) develop on the tissue of dead plants and remain in the soil (Photo sourced from Jangir et al., 2021).

2.6 *Secreted in Xylem* (*SIX*) genes

2.6.1 General background

Plant pathogenic fungi discharge effectors which are small secreted proteins essential for plant infection to arise by influencing plant immunological responses and other signal transduction activities. To allow the pathogen to proliferate, they engage with particular host regulators either in the apoplast or within host cells (Hogenhout et al., 2009; Guo et al., 2014; Franceschetti et al., 2017; Maldonado et al., 2018). An effector group known as *Secreted in Xylem* (*SIX*), were found to be contributing to the virulence of *Foc* and crucial for host-specific pathogenicity during *Foc*-banana infection (Czislowski et al., 2021). *SIX* genes were formerly considered to be exclusive to *Fol*, but their homologs have now been also identified in other formae speciales. Homologs are genes that normally have a common evolutionary origin. Besides, *SIX* genes' presence or absence, as well as their sequence variation have been utilized as new method to discriminate distinct *Fusarium oxysporum* f.sp. and also their races (Fraser-Smith et al., 2014; Jenkins et al., 2021).

SIX genes have been employed as a diagnostic marker due to their variations in their arrangement among members of distinct formae speciales and races (Lievens et al., 2008; 2009). This was presented in a few previous studies, for instance, Chakrabarti et al. (2011) stated that *SIX6* gene was employed as a molecular marker to distinguish pathogenic *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*) isolates specific to cotton from non-pathogenic isolates obtained from the same geographic region in Australia. According to Jangir et al. (2021), the presence, copy number, and sequence variability of the *SIX1* effector gene in *Foc* were used to distinguish between race 1 isolates that infects banana cv. Gros Michel and race 4 isolates that are harmful to Cavendish banana.

Likewise, Maldonado et al. (2018) stated that sequencing of *SIX* genes found in banana infecting *Foc* isolates revealed that *Foc*1 has *SIX1, SIX6, SIX9* and *SIX13* genes whereas *SIX1, SIX2, SIX6, SIX7, SIX8*, *SIX9* and *SIX13* genes were found in *Foc*4. On top of that, a few studies have distinguished three races of pathogenic *Fol* that infects tomato using the specific sets of *SIX* genes found respectively in each of them. In race 1, three *SIX* genes (*SIX1, SIX3, SIX4*) were identified. Whereas race 2 has two (*SIX1, SIX3*) genes and race 3 has only *SIX1* gene present in their genome (Rep et al., 2004; Houterman et al., 2008, 2009; Van Der Does et al., 2008; Lievens et al., 2009; Takken and Rep, 2010; Kang et al., 2014; Jangir et al., 2021).

2.6.2 Structure and location of *SIX* genes in fungal genome

All the 14 *SIX* genes in *Fol* (except *SIX13*) are found on the lineage specific (LS) chromosome 14 (Schmidt et al., 2013; Rocha et al., 2015; Jenkins et al., 2021). Fundamentally, *SIX* genes harbour two miniature inverted transposable elements (MITEs), known as mimp (miniature impala; sized 220 nucleotides) and mFot5 (miniature Fot5 transposon) that are distributed differently (Figure 2.5).

According to Jangir et al. (2021), class II TEs such as mimp and mfot5 are found in the promoter region of *SIX* gene. Some *SIX* genes might be trapped between the TEs' internal resolution (IR) and eventually transposed together. The transposition activity may be the cause of variations in the *SIX* profiles observed among the several formae speciales of *Fusarium oxysporum* species complex (FOSC).

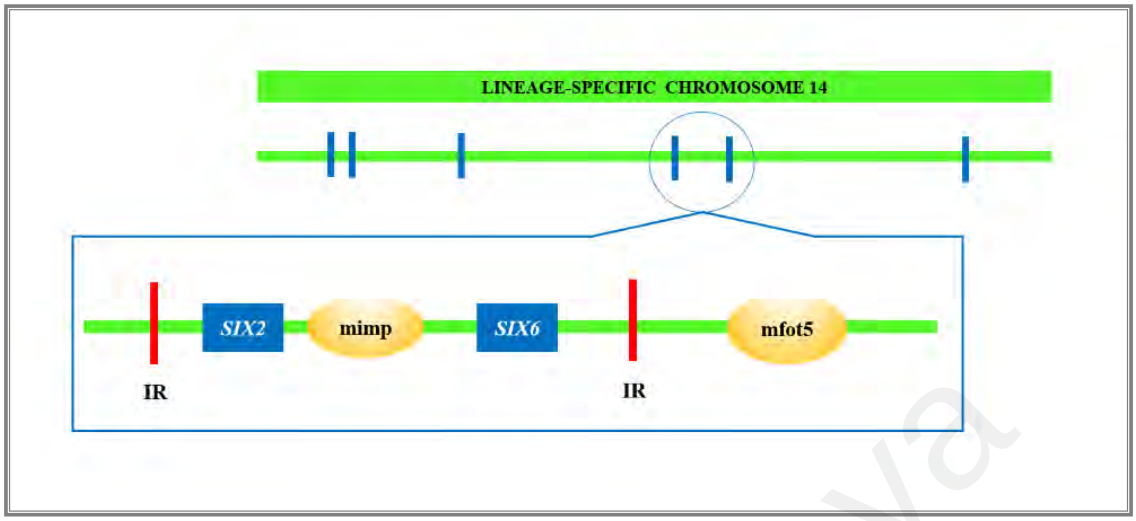


Figure 2.5: The representative arrangement of *SIX* genes between the IR of transposable elements in lineage-specific chromosome 14. IR, Internal resolution; mimp, miniature impala
(Photo modified from Jangir et al., 2021)

2.6.3 Roles of *Secreted in Xylem* (*SIX*) genes

Effectors are used as molecular weapons by plant pathogens to manipulate host immunity and facilitate colonization (An et al., 2019). The fundamental knowledge on virulence-associated proteins is essential to understand plant-microbe interactions (Takken and Rep, 2010). There are still unanswered concerns on how the interaction of host-pathogen works in this pathosystem. Presently, there are limited studies focusing on virulence genes' identification in *Fusarium oxysporum* f.sp. cubense (*Foc*) (Widinugraheni et al., 2018). Be that as it may, research on other formae speciales in the *Fusarium oxysporum* species complex (FOSC) has been an eye opener. A few studies have discovered that the pathogenicity and virulence of FOSC are significantly influenced by *Secreted in Xylem* genes.

A total of 14 "*Secreted in Xylem*" (*SIX*) genes were identified in *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) and most of them resided on chromosome 14, a single 'pathogenicity' chromosome. Some of these have been demonstrated to possess specific functions in influencing the virulence and/or avirulence of the *Fol*-tomato pathosystem (Widinugraheni et al., 2018). For instance, *SIX1, SIX3, SIX5, SIX6* code for proteins that contribute to virulence of *Fol* (Srinivas et al., 2019; van der Does et al., 2008). In another study conducted by Thatcher et al. (2012), homologs of *Fol SIX1, SIX4, SIX8* and *SIX9* were identified in the genome of *Fo*5176 isolate that infects *Arabidopsis*. Among them, *SIX1, SIX6, SIX8,* and *SIX9* were also discovered in our present study.

2.6.3.1 *Secreted in Xylem 1* (*SIX1*)

Previous studies have found that *SIX1* trigger resistance in tomato plants, implying its contribution to *Fol*'s virulence (Houterman et al., 2007; van der Does et al., 2008). Homologs of *SIX1* were found in many formae speciales of *Fusarium oxysporum*. This includes *Foc*, *Fol*, *Fusarium oxysporum* f.sp. *melonis* (*Fom*), *Fusarium oxysporum* f.sp. *conglutinans* (*Focon*) and *Fusarium oxysporum* f.sp *pisi* (*Fop*) (van Dam et al., 2016).

Widinugraheni et al. (2018) examined whether *SIX1a* is necessary for TR4's virulence in a bioassay carried out on Cavendish bananas. A *SIX1a* deletion mutant (Δ*SIX1a*) was utilised in this study. They generated a mutant *SIX1a* gene from *Foc*TR4 II5 isolate (*Foc*Δ*SIX1a*) and tested in planta. It was found that the mutants' pathogenicity decreased severely. The ectopic integration of *Foc-SIX1a* gene in the *Foc*Δ*SIX1a* isolate restored virulence to the same level as observed in the wild type. Hence, this study demonstrated that *SIX1a* is necessary for *Foc*TR4 to exhibit full virulence towards Cavendish banana.

In another study, Li et al. (2016) found that the expression of *SIX1* in *Fusarium oxysporum* f.sp. *conglutinans* (*Focon*) significantly increased during infection on cabbage. *Focon*'s *SIX1* deletion and complementation experiments demonstrated the contribution of *SIX1* as a virulence factor against cabbage. In spite of the fact that not all formae speciales possess *SIX1* homolog, these observations are similar with the results obtained in onion, tomato, and cabbage infecting isolates (Rep et al., 2004; Li et al., 2016; Taylor et al., 2016) suggesting that *SIX1* serves general virulence function in *Fusarium oxysporum*.

2.6.3.2 *Secreted in Xylem 6* (*SIX6*)

Houterman and co-researchers discovered the presence of *SIX6* in the xylem sap of tomato plants seven weeks after inoculation in *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) for 2.5 weeks (Houterman et al., 2007; Lievens et al., 2009). *SIX6* was found to be expressed in both the early and advanced stages of host infection, with its active expression dependent on the presence of a viable host cell. To investigate the potential contribution of *SIX6* to virulence, the researchers inoculated ten-day-old susceptible tomato seedlings with either *SIX6* knockout isolates, independent ectopic transformants without a *SIX6* gene deletion, wild-type *Fol* or water as control. The disease scoring that was carried out at 3 weeks post-inoculation revealed a substantial decrease in disease index of the knockout isolates.

The mutant isolate was subsequently transformed with a *SIX6* complementation construct to ascertain that the mutant's reduced virulence was attributable to *SIX6* deletion, and they discovered that the pathogenicity was fully restored. Additionally, gene expression analysis was performed on the knockout isolate and the derived complementants. Both wild-type *Fol* and *SIX6* complementants had *SIX6* amplification

except for the knockout isolate, providing additional proof that it is a genuine knockout. In conclusion, *SIX6* can be regarded as a valid *Fol* effector gene as the mutation diminishes *Fol*'s pathogenicity, which can be recovered by ectopic complementation. Therefore, it has been confirmed that *SIX6* is actively expressed within the plant during infection and is necessary for *Fol*'s complete virulence.

2.6.3.3 *Secreted in Xylem 8* (*SIX8*)

As for *SIX8*, An et al. (2019) knocked out this gene to investigate its role within *Foc* TR4. The virulence of *SIX8* gene was analysed by inoculating Cavendish banana plantlets with wild type and mutant isolates. The disease symptoms were observed in both wild type-inoculated and mutant-inoculated plantlets after 4th and 5th week. Through observation, it was found that wild type-inoculated plants started showing symptoms after the 3rd week and the symptoms aggravated with the inoculation time. The plantlets inoculated with mutant isolate, on the other hand, exhibited few disease symptoms after being treated for five weeks while maintaining healthy leaves, rhizomes and pseudo stems, denoting *SIX8* mutant isolate's significant virulence impairment.

Furthermore, analysis of the rhizome and pseudostem of the plantlets unveiled that the mutant *SIX8* gene was unable to colonize the banana roots in the same manner as wild type isolate. This finding indicates that *SIX8* is crucial in regulating infection and the early stage of *Foc*TR4's biotrophic development. Complement mutants were constructed to determine *SIX8*'s role in virulence. This resulted in a partly restoration of virulence, underlining that *SIX8* is necessary *Foc*TR4's virulence towards Cavendish banana (An et al., 2019).

2.6.3.4 *Secreted in Xylem 9* (*SIX9*)

SIX9 is another effector protein that has gained attention for its virulence activity. Sakane et al. (2023) conducted a study on *Fusarium oxysporum* f.sp. *cepae* (*Focp*) which infects Welsh onion and reported that *SIX9* was conserved in *Focp* isolates obtained from both bulb onion and Welsh onion. Notably, *SIX9* in *Focp* exhibited high virulence on Welsh onion. It was expressed in plants after inoculation suggesting that *SIX9* is crucial to the infection of *Allium* species by *Focp*. Moreover, Haapalainen et al. (2022) presented evidence indicating a correlation between the level of *SIX9* expression in *Focp* and the strength of virulence towards onions. Therefore, it has been established that *SIX9* plays an essential role in pathogenicity of the *Focp* species.

2.6.4 Importance of studying *SIX* genes in *Foc*

The interaction between pathogen and plant genotypes, as well as environmental factors impacts significantly on the progression of Fusarium wilt in bananas (Groenewald et al., 2006). As a typical necrotrophic fungus, *Fusarium oxysporum* f.sp. *cubense* can persist in soil for extensive stretches as thick-walled chlamydospores prior to infecting banana (Di Pietro et al., 2003). Due to the variety of formae speciales and races in FOSC, pathogenicity assays are tedious and difficult to discriminate between isolates that are pathogenic and non-pathogenic (Recorbet et al., 2003). Methods that make use of specific gene sequences linked to pathogenicity such as *SIX* genes can be utilized to overcome the challenges mentioned above (Recorbet et al., 2003; van der Does and Rep, 2007; Lievens et al., 2008; van Dam et al., 2018).

In such manner, *SIX* genes can operate as a specific and sensitive diagnostic marker to detect this disease during early infection stages. By identifying unique pathogenicity factors in the pathogenic isolates of *Foc,* researchers can gain a better comprehension on how this pathogen infects bananas as well as a source for quickly recognizing isolates that are pathogenic (Ghag et al., 2015). Therefore, improved understanding of the *SIX* gene's role in *Fo*'s virulence and variability has paramount implications for the development of a molecular framework and disease management approaches. Mohd-Yusuf et al. (2019) highlighted that recent findings in genetic approaches to comprehend *Foc*-banana interactions have started to reveal insights into the potential outcomes of investigating novel techniques for resistant cultivar production. Hence, research related to interaction of *SIX* genes in *Fusarium oxysporum* f.sp. *cubense* and banana will bring better insights regarding the components on the reaction of host plant towards the fungal infection, perhaps prompting disclosure of proficient approaches to combat banana Fusarium wilt disease.

2.7 Molecular detection of plant pathogens

Early detection of plant diseases before any symptom appearance is a vital source of information to implement proper strategies for pest management and disease control measures so that the disease can be curbed from developing and spreading further (Sankaran et al., 2010). There are a few approaches for detecting plant diseases that involve conventional methods, direct methods and indirect methods. The conventional method is mainly conducted through visual examination. It involves interpreting the disease symptoms visually such as blight, galls, spots, tumours, rots or wilts followed by pathogen isolation and microscopy technique (Ray et al., 2017). The direct methods utilize the immunology- and polymerase chain reaction (PCR)-based methods whereas the indirect methods make use of stress- and biomarker-based techniques. The PCR-based

methods are commonly used, and they are relatively fast approach that enables highly accurate and sensitive detection of specific target nucleic acids.

Of these, reverse transcription quantitative real-time PCR (RT-qPCR) is a technological advancement that has enabled researchers all around the world to develop applications that are powerful and innovative. It has numerous advantages over other approaches for measuring gene expression. This technique is employed to measure the prevalence and activity of functional gene markers in the environment, and it finds widespread use in microbial ecology. In order to record the amplicons' build up in 'real-time', fluorescence detection technology is paired up with the conventional end-point detection PCR during each PCR amplification cycle (Smith and Osborn, 2009). qPCR works in a similar way to end-point PCR, with multiple amplification cycles where the first step is denaturation of DNA template. Next, oligonucleotide primers targeting specific sequences are annealed, and then a thermostable DNA polymerase extends a complementary strand from each annealed primer, causing the amplicon numbers to increase exponentially during PCR. Unlike end-point PCR, however, a 'real-time' record for the rise in amplicon numbers is taken during PCR by detecting a fluorescent reporter that reveals accumulation of amplicons during each cycle. The intercalating SYBR green assay and the *Taq*man probe methods are the two extensively utilised reporter systems in this RT-qPCR approach (Taneyhill and Adams, 2008).

a) SYBR green assay method

SYBR green intercalates across adjacent base pairs to bind to all double-stranded DNA (Taneyhill and Adams, 2008). After exposure to light stimulation, a fluorescent signal is emitted when it binds to DNA. The number of amplicons rises after each PCR cycle, resulting in fluorescence increase. Since SYBR green binds to all double stranded DNA,

employing highly specific primer pairs that accurately binds to the target sequence is critical to preclude generating products that are non-specific which would contribute to the presence of fluorescence signal, causing the target to be overestimated. To make sure the formation of desired product only, primer concentrations that are optimized extensively might be required to be used in SYBR green based qPCR tests (Smith and Osborn, 2009). Figure 2.6 briefs the SYBR green assay method.

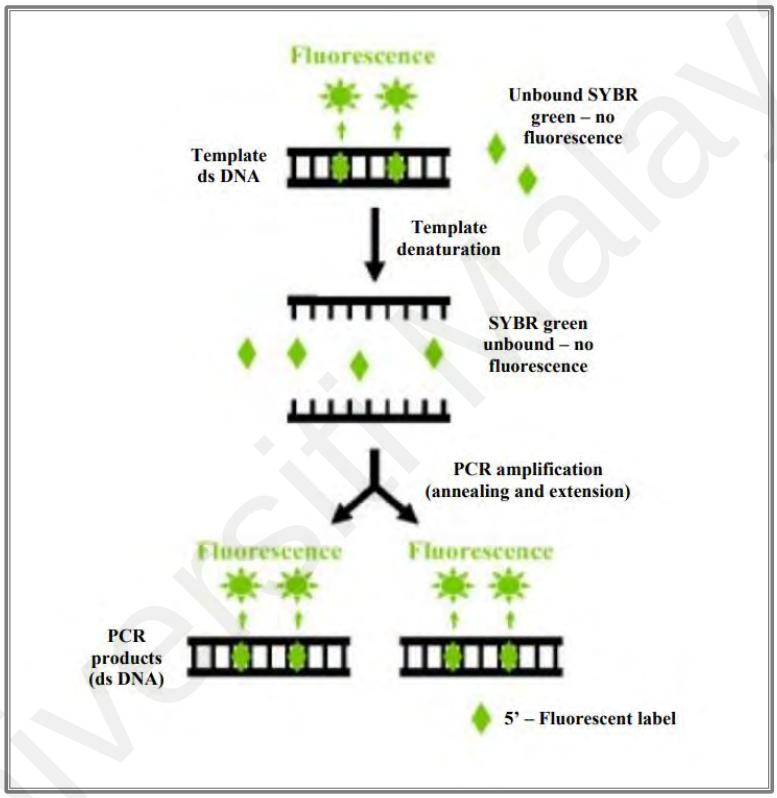


Figure 2.6: SYBR green assay. SYBR green fluorescent dye binds to all double stranded DNA and emits a fluorescence signal. SYBR green does not glow when it is free. The corresponding fluorescent increase is utilised to quantify amplification of template in each cycle (Photo sourced from Smith and Osborn, 2009).

b) Taqman probe-based method

The *Taq*Man probe approach works by utilizing a probe that is fluorescently labelled which can hybridize to a conserved area within the targeted amplicon sequence (Taneyhill and Adams, 2008). The 5' end of the *Taq*Man probe is labelled fluorescently, while a quencher molecule is present at the 3' end. As a consequence of fluorescent resonance energy transfer, the quencher molecule's closeness to the fluorophore on the probe hinders it from fluorescing. The target sequence is attached to the specific primers and intact probe during annealing stage of each PCR cycle. During the subsequent template extension, 5' exonuclease activity of the *Taq* polymerase enzyme cleaves the fluorophore from the *Taq*Man probe, leading to the detection of fluorescent signal. This occurs because the fluorophore is no longer in close proximity to the quencher. The fluorophore's release and build-up throughout each cycle's extension stage are thus used to determine the template's amplification (Figure 2.7) (Smith and Osborn, 2009).

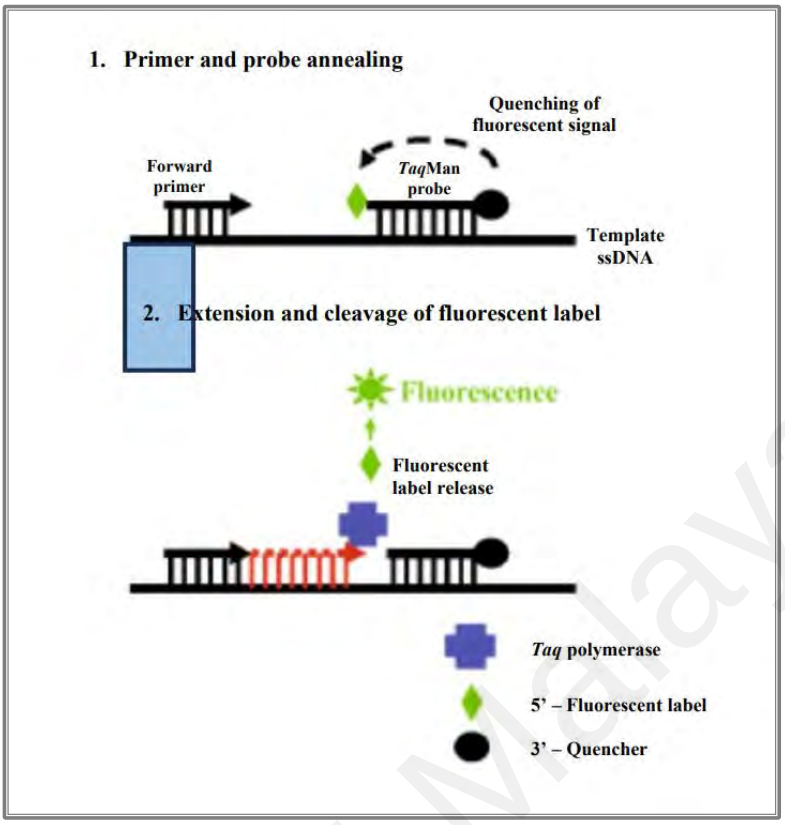


Figure 2.7: *Taq*Man (5' nuclease) assay using *Taq*Man® probes. Both the *Taq*Man probe and primers attach to the DNA template during the annealing step. The energy is passed between the quencher and the reporter while the *Taq*Man probe is intact, therefore no detection of fluorescent signal. The probe labelled 5' nucleotide is cleaved by 5' exonuclease activity of *Taq* polymerase releasing the reporter from the probe as a new strand is produced. Once the probe and reporter are no longer in close proximity, the fluorescent signal from the probe is detected. The amplification of the template is quantified by the fluorescence's proportional rise (Photo sourced from Smith and Osborn, 2008).

2.8 Whole Genome Sequencing

Due to the advancements in science, the evaluation of genetic diversity experienced a revolution over a decade ago with the advent of high-throughput sequencing tools (Goodwin et al., 2016). These technologies enable the rapid and affordable sequencing of thousands to millions of loci, resulting in substantially higher marker density than that was possible with earlier technologies. As a result, researchers may now sequence novel genomes using techniques like whole genome sequencing. The comprehensive method of whole genome sequencing (WGS) grants for the analysis of entire genomes due to the effectiveness of modern sequencers to generate massive volumes of data (https://www.illumina.com/). Generally, WGS often offers a base-by-base, high resolution view of the genome. It also finds potential causal variations for additional follow-up research of gene expression and regulation mechanisms. It captures both large and small variants that targeted techniques might overlook (https://www.illumina.com/).

2.8.1 Types of Whole Genome Sequencing

De novo whole-genome sequencing (WGS) and the whole genome resequencing (WGR) are the two subcategories of whole genome sequencing. WGS aims to generate the first-ever assembly of complete genome sequences (Fuentes-Pardo and Ruzzante, 2017). Figure 2.8 gives a quick rundown of WGS sequencing technique.

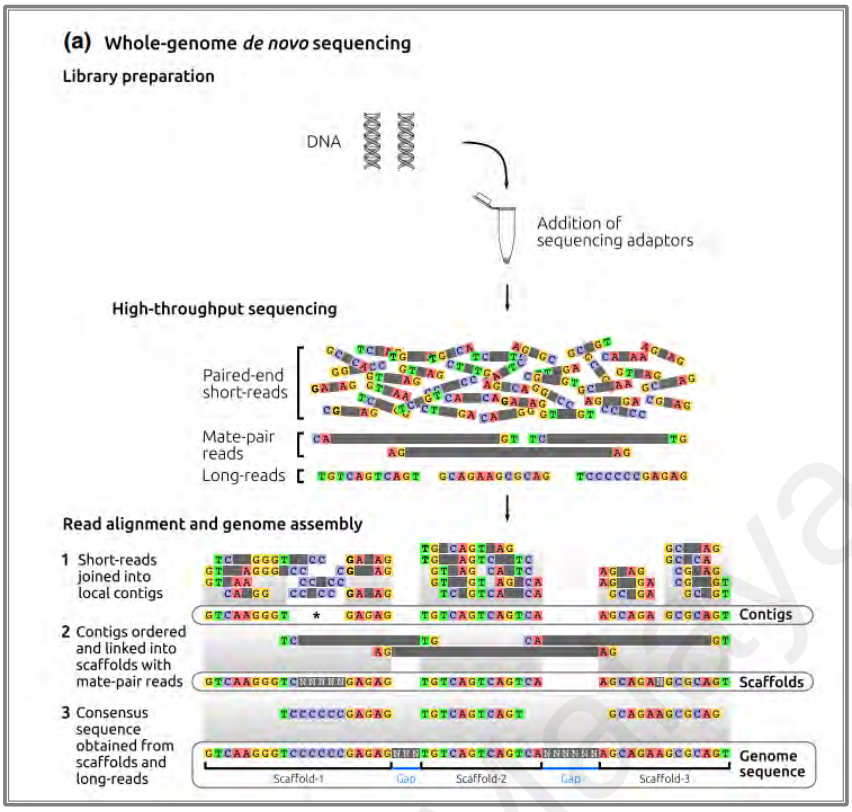


Figure 2.8: Whole genome *de novo* sequencing (Photo sourced from Fuentes-Pardo and Ruzzante, 2017)

High-quality genomic DNA is first broken up into smaller fragments for library preparation, which includes adding sequencing adaptors to the DNA fragments. High-throughput sequencing is used to produce paired-end short reads (~100 bp) from libraries with various insert sizes in order to maximize genome coverage. The sequence pool can also be complemented by long-read sequences (~2–10 kb) (Fuentes-Pardo and Ruzzante, 2017). The first step to read alignment is the construction of local contigs, which are the sequences made up of overlapping DNA fragments. Contigs are then oriented and linked together by mate-pair readings, creating longer sequence stretches known as scaffolds. The assembly of repetitive regions is further aided by the lengthy reads. The end result of a genome assembly is typically a consensus sequence, often comprised of a series of contiguous scaffolds interspersed with gaps (Fuentes-Pardo and Ruzzante, 2017).

On the other hand, WGR aims to analyse genomic differences between individuals or populations. In this approach, the use of a reference genome is essential for read mapping and identification of variants (Ekblom and Wolf, 2014). When implementing a WGR approach, geneticists are most likely to face challenges when there is no reference genome available for the species of interest. This situation justifies resorting to the genome sequence of a closely related species (Lamichhaney et al., 2012; Dennenmoser et al., 2017). However, caution is advised because even across closely related species, changes in genomic organization, such as structural polymorphism or copy number variation might exist (Ekblom and Wolf, 2014). Figure 2.9 gives a quick rundown of WGR sequencing technique.

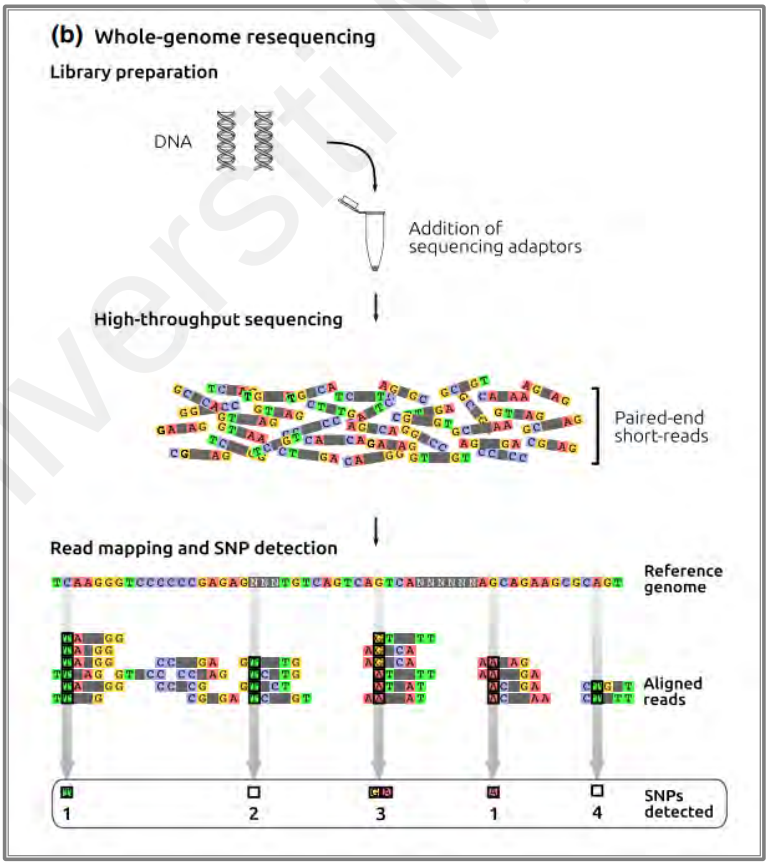


Figure 2.9: Whole genome resequencing (Photo sourced from Fuentes-Pardo and Ruzzante, 2017)

In this approach, high-quality genomic DNA is fragmented to prepare a library, wherein sequencing adaptors are attached to the DNA fragments. High-throughput sequencing is used to produce paired-end short reads from the DNA library that are about 100bp long. Based on sequence similarity, short reads are mapped onto the species reference genome. According to Fuentes-Pardo and Ruzzante (2017), a SNP is discovered when nucleotide in the reads differs from the specific base found at a particular site in the reference genome. Some SNPs may be lost due to absence in the reference genome, heterozygosity in the subject reads or loss due inadequate coverage during filtering.

2.8.2 Next Generation Sequencing (NGS)

Next generation sequencing (NGS), also known as massively parallel or deep sequencing is a DNA sequencing technology that has revolutionised genetic research. The prime purpose of NGS in microbiology is to replace the conventional morphological, staining and metabolomic characterization of pathogens with a genomic definition. The genomes of pathogens provide information about who they are, how sensitive they are to drugs, and how they interact with other pathogens. These characteristics can be exploited to identify the origins of infection outbreaks (Behjati and Tarpey, 2013). In addition to sequencing whole genomes, NGS can be combined with DNA capture techniques for specialised analysis of particular genomic areas, particular genes or the entire exome. Apart from that, NGS can also be used to sequence RNA (Reis-Filho, 2009).

Rapid advancements in DNA sequencing technology have resulted in significant cost reductions, as well as increases in the accuracy and throughput. The world is being inundated with a deluge of genetic data every day as more and more organisms are being sequenced. Due to a revolution in sequencing technology, progress in genomics has been advancing steadily. Large scale studies in transcriptomics, metagenomics, exomics, and

epigenomics are also becoming a reality. These studies offer direct application benefits in addition to knowledge for fundamental research. These data are being used by researchers in a variety of sectors to improve livestock, crop yields as well as diagnosis and treatment of cancer and other complex diseases (Liu et al., 2012).

2.8.2.1 Illumina technology

Illumina stands out as the predominant player in the second-generation sequencing market. The Illumina sequencing technique involves a "bridge amplification" method, where DNA molecules act as substrates for multiple amplification synthesis cycles on a solid support, typically a glass slide. This slide carries oligonucleotide sequences complementary to a ligated adapter. Through numerous rounds of amplification, the oligonucleotides on the slides are strategically arranged, generating clonal "clusters", each compromising around 1000 copies of every oligonucleotide fragment (Slatko et al., 2018). There can be millions of parallel cluster reactions supported by each glass slide. The synthesis reactions are tracked by introducing proprietary modified nucleotides, each labelled with a distinct fluorescent tag that corresponds to one of the four DNA bases. In each reaction, the nucleotides also serve as synthesis terminators, and upon detection, they are released to enable additional synthesis. This process is iterated for 300 or more rounds. Unlike camera-based imaging, the utilisation of fluorescence detection expedites the detection time as it allows for direct imaging (Slatko et al., 2018).

2.8.3 Third Generation Sequencing (TGS)

Third generation sequencing (TGS) has established itself in biology as a method for studying genomes, metagenomes, and transcriptomes at unprecedented resolution. This technology has been applied to produce exceptionally accurate *de novo* assemblies of hundreds of microbial genomes. Additionally, it has facilitated the creation of highly contiguous reconstructions for dozens of plant and animal genomes (Lee et al., 2016). The two TGS technologies that are most prevalent are PacBio sequencing and Nanopore sequencing.

2.8.3.1 Pacific Biosciences (PacBio) sequencing

PacBio stands as the pioneer in third-generation sequencing, being the first commercially available long-read high-throughput sequencing technology (Tedersoo et al., 2021). This platform leverages the single-molecule real-time (SMRT) technology (Xiao and Zhao, 2020). The sequencing method offers highly contiguous *de novo* assemblies, enabling the characterization of structural variations in individual genomes and the filling of gaps in existing reference assemblies.

Besides, PacBio transcriptome sequencing aids in the discovery of novel genes and isoforms of recognized genes. This capability stems from its ability to read full-length transcripts or significant-length fragments, providing a more comprehensive view of the transcriptome. Moreover, it offers data that can be utilized to precisely detect base alterations like methylation (Rhoads and Au, 2015). Briefly, PacBio's SMRT technology employs eight million zero-mode waveguides (ZMW) that are microwells containing DNA polymerase. These ZMWs capture signals generated by the incorporation of fluorescently labeled nucleotides during the DNA replication of SMRTbell templates. The detection system creates a continuous long read from each ZMW by recording a

movie of light pulses, which are then translated into base calls (https://genomics.umn.edu/).

2.8.3.2 Nanopore sequencing

The next TGS technology is nanopore sequencing by Oxford Nanopore Technologies (ONT). In this method, signal disruptions of nucleotide oligomers are captured as they move through nanopores to transport DNA molecules (Tedersoo et al., 2021). It makes use of electro-resistant membranes with embedded nanopores where each nanopore has a unique electrode that connects to a channel and sensor chip that detects the passage of electricity. A distinctive signal is created when there is a disruption in the current in a nanopore. Base calling algorithms are then used to decode this signal in real-time in order to ascertain the DNA or RNA sequence (https://genomics.umn.edu/). These characteristics enable the detection of hundreds of kilobases in a single continuous read and even ultra-long reads (ULRs) longer than 300kb and up to one million bp (Midha et al., 2019).

CHAPTER 3: METHODOLOGY

This research involves three parts including fungal works, molecular works and whole genome sequencing (Figure 3.1). Briefly, Malaysian fungal isolates, *Foc*1_C2HIR and *Foc*4_C1HIR were cultured, DNA extracted and subjected for three different purposes; (a) Whole genome sequencing (b) Detection of target (*SIX*) genes (c) Bioassay experiment. Following the bioassay experiment, the plant samples were harvested at different time points and used for gene expression studies. The 5th week post-inoculation plants were assessed for disease symptoms to determine the fungal pathogenicity level. The bioassay experiment was ethically conducted in a greenhouse environment (CBC Reference No: SEP/24/19/C/44).

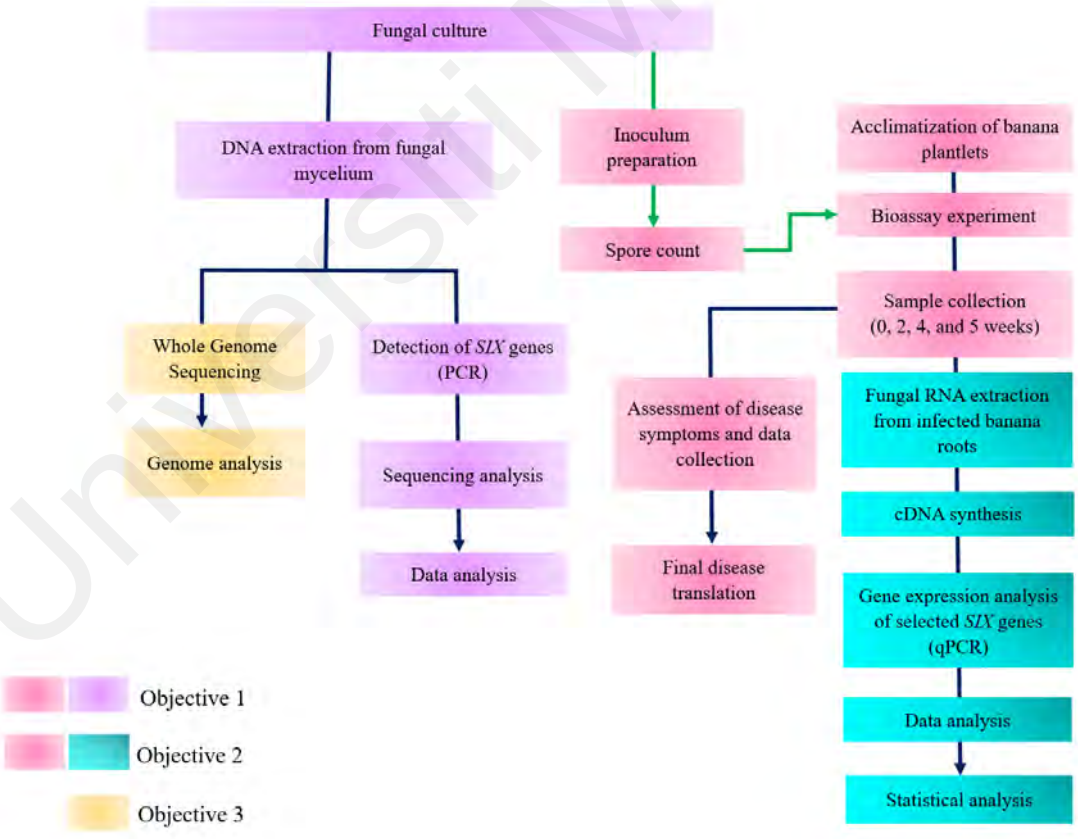


Figure 3.1: Overview of research methodology applied in this study

3.1 Materials

3.1.1 Plant Materials

3-month-old rooted tissue culture-generated *Musa acuminata* cv. 'Berangan' plantlets were purchased at Malaysian Agricultural Research and Development Institute (MARDI). Healthy plantlets with at least 3 to 5 leaves, a minimum height of 20cm, and stem diameters between 1.0 to 1.5cm were selected for infection studies.

3.1.2 Fungal isolates

Fusarium oxysporum f.sp. *cubense* (*Foc*) Race 1 (C2HIR isolate) and Race 4 (C1HIR isolate) of Malaysian origin were from our collection at the Universiti Malaya's PhytoMycology Laboratory. The fungus was kept at -80°C as a microconidial suspension in 30% glycerol stock.

3.2 Methods

3.2.1 Fungal culture

Starter cultures were prepared by transferring isolates from the glycerol stock onto petri dishes of full strength Difco™ Potato Dextrose Agar (PDA) (BD, France) supplemented with 500µl of streptomycin sulphate (50µg/ml) (Sigma Aldrich, Germany) for every 500ml of media. A total of 100µl from each isolate of *Foc*1_C2HIR and *Foc*4_C1HIR were inoculated on PDA plates respectively according to the labels. The plates were kept in dark condition at 25±2°C for seven days. The isolates were confirmed to be *Fusarium oxysporum* f.sp. *cubense* after observing their morphological characteristics and through molecular analysis (refer to 3.2.8 and 3.2.13).

3.2.2 Preparation of spore suspension culture

Spore suspension cultures were made by inoculating 1L of Difco^{TM} Potato Dextrose Broth (PDB) (BD, France) with one loop full of mycelia previously grown on PDA. The spore suspension was incubated in dark condition and shaken twice daily at room temperature (25±2°C). On the 7th day after inoculation, the spores were counted using a hemocytometer, and an appropriate dilution using sterile distilled water was made to achieve a final concentration of 1 x 10^6 spores/ml. A total of 4L of suspension culture was prepared for each set of treatments.

3.2.3 Fusarium wilt bioassay

Tissue culture rooted plants were potted in 12 x 9cm^2 pot with a 3:1 ratio mix of sterilized soil and sand. Acclimatization took place at ambient temperature 28±2°C in the greenhouse. A total of 48 'Berangan' plantlets that met the specific criteria described in section 3.1.1 were carefully uprooted for inoculation. These 48 plants were divided and subjected to three treatments as follows:

1. Treatment 1 (Control) : Plants were immersed in sterile distilled water.
2. Treatment 2 : Plants were immersed in *Foc*1 suspension culture.
3. Treatment 3 : Plants were immersed in *Foc*4 suspension culture.

Before *Foc* inoculation, plant roots were rinsed with tap water to eliminate any soil residues. Next, the plant roots were immersed accordingly in sterile distilled water, *Foc*1 suspension and *Foc*4 suspension for 2 hours. After that, the infected plants were replanted in their original soil with the proper labelling. A double-tray setup was used to relocate the plants and kept under the same conditions in the greenhouse. In the double-tray apparatus system, two trays were used, a small tray measuring 43 x 29 x 9cm^3 that fits firmly with another larger outer tray measuring 46 x 31x 20cm^3. The plants were irrigated

every other day with tap water and Hoagland solution (Sigma-Aldrich, U.S.A.) once a week. Sterile distilled water served as the inoculation medium for the negative control plants.

3.2.4 Sample collection and experimental design

The root samples of the treated plants were collected at four different time points; 0 day, 2 weeks, 4 weeks and 5 weeks of post-infection for molecular study. These tissue samples were flash frozen in liquid nitrogen before being kept at -80ºC for further use. The experimental design for the sample collection is outlined in Table 3.1.

Table 3.1: Experimental design of post-inoculation samples collection at four different time points. A total of 12 plants were collected at each time point that consists of four plants from each treatment.

Time / Treatment	0 day	2 weeks	4 weeks	5 weeks	Total
Treatment 1 (control)	4	4	4	4	**16**
Treatment 2 (*Foc*1)	4	4	4	4	**16**
Treatment 3 (*Foc*4)	4	4	4	4	**16**
Total	**12**	**12**	**12**	**12**	**48**

3.2.5 Assessment of disease symptoms and data collection

The external symptoms of Fusarium wilt were evaluated visually. At the end of 5th week post-inoculation, the plants were scored according to the degree of infection of each symptom. The degree of leaf wilting and yellowing was appraised using a 5-point scale, and this value was used to determine the Leaf Symptom Index (LSI) of the cultivar (Table 3.2).

In order to assess the internal symptoms, infected plants were uprooted carefully from their pots and their roots were washed to remove soil residues. The rhizomes were then vertically cut into half to observe any brownish or dark discoloration. Scores were assigned for the intensity of rhizome infection using an 8-point scale as outlined in Table 3.2, to quantify the level of discoloration, thereby calculating the Rhizome Discoloration Index (RDI). These LSI and RDI were utilized to calculate the disease severity index (DSI), which was employed to determine the susceptibility or resistance level of the tested cultivar.

Table 3.2: Leaf Symptom Index (LSI) and Rhizome Discoloration Index (RDI) scale values used in the evaluation of plants' response against *Foc* infection (Brake et al., 1995; Mak et al., 2004).

	Score	Symptoms
Leaf symptom index (LSI)	1	No streaking or yellowing 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 the leaves
	5	Dead plant
Rhizome discoloration index (RDI)	1	No discoloration of tissue or 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

3.2.6 Final Disease Translation

The final disease translation for the pathogenicity test experiment was determined using Disease Severity Index (DSI) utilising data produced from the LSI and RDI (Mak et al., 2004). DSI is computed by considering LSI and RDI scores, which gauges the disease response of infected plants based on leaf yellowing and rhizome discoloration, respectively. The formula for DSI calculation is presented below.

$$\text{DSI} = \frac{\Sigma\,(Number\ on\ scale\ x\ Number\ of\ seedlings\ in\ that\ scale)}{\Sigma\,(Number\ of\ treated\ seedlings)} \quad (3.1)$$

The resulting DSI value is associated with one of the four classifications outlined in Table 3.3. If either the LSI or RDI indicates susceptibility, the cultivar is categorized as susceptible to that particular isolate. Conversely, the cultivar was only considered to be tolerant if one of those scores indicate tolerance while the other suggests resistance. When both disease indications are resistant, then the cultivar was deemed resistant.

Table 3.3: 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 2.0	Between 1.1 and 3.0	Tolerant
Between 2.1 and 3.0	Between 3.1 and 5.0	Susceptible
Between 3.1 and 5.0	Between 5.1 and 8.0	Highly susceptible

3.2.7 Fungal recovery assay

Fungal recovery assay was carried out to identify the colonization of *Foc* in the vascular tissues and recover them. The root and stem samples of one banana plant per treatment were collected at two weeks post-inoculation to assess the colonization potential of *Foc* in banana. The samples were surface sterilized for about four minutes using 70% ethanol. The ethanol was then removed, and stems were rinsed twice with sterile water. The tips of each root and stem were cut off and the middle portion was placed on PDA plates containing 50mg/ml streptomycin to inhibit bacterial growth. The *Foc* outgrowth was evaluated after five days of incubation at 25±2℃ in the dark.

3.2.8 DNA extraction

Fungal DNA extraction was conducted using FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA) according to the protocol provided by the manufacturer. The fungal mycelia were scraped, flash frozen and ground into fine powder using liquid nitrogen prior to the extraction. Briefly, 0.5g of ground fungal sample was added to a Lysing Matrix E tube followed by 978µl sodium phosphate buffer, 122µl MT buffer and homogenized for 40 secs. The mixture was centrifuged at 14000 x *g* for 10 mins to pellet the debris and the supernatant was transferred to a clean 2ml microcentrifuge tube. A total of 250µl PPS solution was added to the supernatant, mixed and centrifuged at 14000 x *g* for 5 mins to pellet precipitate. The supernatant was then transferred to a 15ml microcentrifuge tube and mixed with 1ml of binding matrix suspension. The mixture was mixed well for 2 mins and placed on a rack for 3 mins to allow settling of silica matrix. A total of 500µl of the supernatant was removed and the remaining amount was transferred to a SPIN™ filter and centrifuged at 14000 x *g* for 1 min. The catch tube was emptied followed by an addition of 500µl of SEWS-M solution and centrifugation at

14000 x *g* for 1 min. The catch tube was emptied and centrifuged again for 2 mins to dry the matrix of residual wash solution. Later, the catch tube was replaced, and the spin filter was air dried at room temperature for 5 mins. The binding matrix was gently resuspended in 50µl of DES solution and centrifuged at 14000 x *g* for 1 min. The DNA was stored at -20ºC for further use.

3.2.9 RNA extraction

Prior to extraction, all solution was rendered RNase free by treating with diethyl pycarbonate (DEPC) and the consumables were sterilized for RNA work purposes. In a cooled mortar and pestle, approximately 0.5g of fungal infected banana roots were ground into fine powder with liquid nitrogen. A total of 1ml of CTAB buffer (Appendix A1) and 20µl β-mercaptoethanol were pre-heated at 65ºC for 10 mins. It was added to the sample, followed by an equal volume of chloroform: isoamyl alcohol, CHCl3: IAA (24:1), vortexed and centrifuged at 9400 x *g* for 15 mins. The aqueous phase was transferred to a 1.5ml tube. This step was repeated two more times. At final centrifugation, 3M sodium acetate (NaOAc) of 0.1 volume was added to the aqueous solution and the tube was filled with ice-cold absolute ethanol. The mixture in the tube was mixed thoroughly and precipitated for two days at -80 ºC. After two days, the tube was centrifuged at 9400 x *g* for 30 mins and the pellet was washed with 1ml of 70% ice cold ethanol. The mixture was centrifuged again at 9400 x *g* for 7 mins. All centrifugation was carried out at 4ºC. The pellet was then air dried and resuspended with 30µl of DEPC-treated water. The RNA extract was stored at -80ºC prior to quantification and qualification of RNA integrity.

3.2.10 DNase treatment

DNase treatment was conducted on all the RNA samples using DNase I kit (NEW ENGLAND Biolabs® Inc.) based on the protocol provided by manufacturer. The amount of reaction mixture is shown in Table 3.4.

Table 3.4: Working solution for DNase treatment on RNA samples

Components	Volume	Final Concentration
DNase I Reaction Buffer	10µl	1x
DNase I	1µl	2 units
RNA	variable	~10µg/µl
Nuclease free water	to 50µl	-

The mixture was incubated at 37ºC for 10 mins. A total of 1µl of 0.5M EDTA was added to the mixture followed by heat inactivation at 75ºC for 10 mins. The DNase treated RNA samples were stored at -80ºC for further use.

3.2.11 cDNA synthesis

The synthesis of cDNA was carried out on DNase-treated RNA samples using Lunascript® RT Supermix Kit (NEW ENGLAND Biolabs® Inc.) based on the instructions provided by the manufacturer. The amount of reaction mixture is shown in Table 3.5. The mixture was incubated at 25ºC for 2 mins, 55ºC for 10 mins followed by 95ºC for 1 min. The cDNA products were then stored at -20ºC for further use.

Table 3.5: Working solution for cDNA synthesis on DNase-treated RNA samples

Components	Volume	Final concentration
Lunascript RT Supermix	4µl	1x
RNA	variable	~1µg/µl
Nuclease free water	to 20µl	-

3.2.12 Primers for Polymerase Chain Reaction (PCR) and Real-time PCR (qPCR)

The list of primers for race specific PCR amplification of *Foc*1 and *Foc*4 is shown in Table 3.6.

Table 3.6: PCR primers for amplification of *Foc*1 and *Foc*4

***SIX* genes**	**Sequences (5' – 3')**	**Amplicon size**	**Reference**
*Foc*1	**F:** AAT CAT GTT GCC AAC GAC AA **R:** GCT CCT CGA CAT CAC CAT TT	245bp	Zaidi, (2019)
*Foc*4	**F:** CAG GGG ATG TAT GAG GAG GCT **R:** GTG ACA GCG TCG TCT AGT TCC	242 bp	Lin et al. (2009)

Primers for PCR amplification of *SIX* genes were obtained from Czislowski et al. (2018). The list of primers is shown in Table 3.7.

Table 3.7: PCR primers for amplification of *SIX* genes

***SIX* genes**	**Sequences (5' – 3')**	**Amplicon size**
SIX1	**F:** GCC CCT TAT TAT CCC TCC GG **R:** GCT GCG AAT CGA AAT AGA GAAA	923bp
SIX2	**F:** TGC AGA TCT TTA CAT AGC TTT GA **R:** TCG GTT TCT CAT CGT AGC TCT	806 – 810bp
SIX4	**F:** CAC CCT CCT AGA CTT GCC TAA **R:** TTA GGA CTT CGA CTT TGC CG	930 – 937 bp
SIX6	**F:** ACA GCT CAA GAA CAC ATC GT **R:** GCG TTA AGG TTT CCG TAG CC	754bp
SIX7	**F:** CCA GAG TCA GTT ATG TGG TAC A **R:** AGC TTA GCA CCC TTG AGT AAC T	810bp
SIX8a	**F:** ACG TCC TTA TAC CCT CTC AAT **R:** ACC CTT ATA TTG CCG CAC CT	624bp
SIX9a	**F:** ACC CGT TTA TAT GTC AAA TCC T **R:** CCG CCT TTG GAT GGT TTC TT	424bp
SIX9b	**F:** ACC ATC ATC ACT CTG CCA GA **R:** GGC AAT CCC AAT GTC GAG AG	461bp
SIX13	**F:** ATA TGG CCT ACT CAT ATC TTG GG **R:** ATC TGT GTT CTA GGC GGA AAA	1076 – 1082bp

Real-time PCR (qPCR) primers were designed using PrimerQuest Tool, Integrated DNA Technologies (IDT) based on our sequences obtained from *SIX* gene sequencing results. Prior to primer designing, gene sequences were copied and saved in FASTA format. Sequences were then transferred into the software to generate gene-specific primers. The designed primers are outlined in Table 3.8.

Table 3.8: qPCR primers used for gene expression studies

***SIX* genes**	**Sequences (5' – 3')**	**Amplicon size**	**Reference**
TEF1α	**F:** TCG GCT ACA ACC CCA AGG CTG **R:** CGG ACT TGA TCT CAC GCT CCC A	120bp	Poon et al. (2019)
TUB	**F:** CCC CGA GGA CTT ACG ATG TC **R:** CGC TTG AAG AGC TCC TGG AT	68bp	Sutherland et al. (2013)
GAPDH	**F:** CCA GAT CAA GCA GGT CAT CA **R:** GTT GGT GTT GCC GTT AAG GT	106 bp	Sutherland et al. (2013)
qSIX1	**F**: AGG CTT TCG AGC GAC TAA TG **R**: GGT TCT GTG GGC AAG TTC T	119bp	-
qSIX6	**F**: CGC TCC GTT TGC TAC AAT TC **R**: GCA AGG AGT GTC GGT AAT CT	90bp	-
qSIX8a	**F:** CCC TTG TCG AAC GGT AAG TAG **R:** CTG TTA TGC AGG CGA GTA AGA	96bp	-
qSIX9a	**F:** GCG CTA GAT ACT GGG CAT TTA **R:** CTG GGT ATC CGA CAC AAA CA	111bp	-
qSIX9b	**F:** CGC CAG ATA CTG GGC ATT TA **R:** TGC ATT GTC CCA TCT CGT ATC	122bp	-

3.2.13 Amplification of *Foc*1, *Foc*4 and *SIX* genes by PCR

PCR using *Foc*1 and *Foc*4 genomic DNA as template was performed using race specific primers as detailed in Table 3.6. Subsequently, PCR using *Foc*1 and *Foc*4 genomic DNA as template was performed using *SIX* gene primers specified in Table 3.7 to confirm the presence of *SIX* genes. Table 3.9 shows the working solution for PCR.

Table 3.9: Working solution for PCR

Components	Volume	Initial concentration	Final concentration
GoTaq® Green Master Mix	10µl	2x	1x
Forward primer	0.5µl	10µM	0.25µM
Reverse primer	0.5µl	10µM	0.25µM
DNA template	1µl	variable	~100 ng/µl
Nuclease free water	8µl	-	-

PCR amplification was conducted in a 20µl reaction volume using the Veriti 96-well thermal Cycler (Applied Biosystems, U.S.A). The amplification followed the cycling conditions outlined in Table 3.10.

Table 3.10: PCR cycling parameters

Step	Temperature	Duration	Cycles
Initial denaturation	95ºC	2 mins	1
Denaturation	95ºC	30 secs	35
Annealing	53ºC to 56ºC	30 secs	
Extension	72ºC	1 min	
Final extension	72ºC	5 mins	1

PCR products were analyzed through agarose gel electrophoresis. A total of 5µl of PCR products were loaded alongside 5µl GeneRuler of 100 bp DNA ladder (Thermo Fisher Scientific, USA) on 1% (w/v) agarose gel stained by GelRad (TransGen Biotech, China). An ultraviolet transilluminator (Bio-Rad ChemiDoc^{TM} MP System) was used to observe the gel after it had electrophoresed at 100V for 30 mins. PCR products were purified using QIAquick PCR Purification KIT (Qiagen, USA) according to manufacturer's protocol and sent for sequencing to a service provider (Apical Scientific Sdn. Bhd.).

3.2.14 Sequencing analysis of PCR products

All sequenced results were analyzed using BLAST software (https://blast.ncbi.nlm.nih.gov) available in National Centre for Biotechnology Information (NCBI) database. Sequence alignment was performed using BioEdit Sequence Alignment Editor software (https://bioedit.software.informer.com).

3.2.15 Real-time PCR (qPCR)

Real-time expression profiles of *SIX* genes involved during *Foc* infection were analyzed using cDNA of fungal infected banana root samples from treatment 2 and treatment 3. Fungal specific endogenous genes, *TEF1α, TUB* and *GAPDH* were initially shortlisted to be used as reference genes. Primer efficiency test was carried out on all the primers (Table 3.8) to test the efficiency and specificity of the primers. Additionally, a stability test using Delta CT method (analysis of most stable genes by lowest standard deviation) was conducted for reference genes to confirm their stability across different treatments and time points. Later, the best endogenous genes were selected based on their efficiency, specificity and stability. Real-time analyses were performed with Applied

Biosystems ViiA 7 Real Time PCR System using SYBR green approach. Table 3.11 shows the working solution for qPCR reaction.

Table 3.11: Working solution for qPCR reaction

Components	Volume	Initial concentration	Final concentration
2x qPCRBIO SyGreen Blue Mix	10µl	2x	1x
Forward primer	0.8µl	10µM	0.4µM
Reverse primer	0.8µl	10µM	0.4µM
cDNA template	1µl	variable	1µg/µl
Nuclease free water	7.4µl	-	-

All the qPCR reaction was carried out in a 20µl reaction volume using 2X qPCRBIO SyGreen Blue Mix (PCR Biosystems, U.S.A). The reaction mixture was incubated in 8-tube strips at 95ºC for 2 mins followed by 40 cycles of 95ºC for 5 secs and 60ºC for 30 secs. Dissociation curves and cycle threshold values of all target genes were established at the end of PCR cycle at 95ºC for 15 secs, 60ºC for 1 min, followed by 95ºC for 15 secs. All reactions were run in triplicates for four biological replicates.

The expression levels were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). The most stable endogenous gene was used in this study as a reference to normalize the expression data and evaluate the response of target genes in the highly susceptible *Musa acuminata* cv. 'Berangan'.

3.2.16 Statistical Analysis

The data generated from gene expression analysis were subjected to statistical analysis of one-way ANOVA and Tukey HSD post-hoc test. Statistical significance of all terms was asserted at the 95% confidence interval ($p<0.05$). The analyses were conducted using IBM SPSS Statistics 26.0 software.

3.2.17 Whole genome sequencing and analysis

3.2.17.1 Raw reads processing, quality filtering and genome assembly

The extracted genomic DNA (gDNA) of *Foc*1_C2HIR and *Foc*4_C1HIR were sent for commercial whole genome sequencing by a service provider (Apical Scientific Sdn, Bhd.). The gDNA samples were prepared into sequencing libraries using NEBNext® Ultra™ IIDNA Library Prep Kit, quantified, pooled and sequenced with an Illumina Novaseq6000 sequencer (150-bp paired-end setting). The raw data obtained for both genomes were assessed for quality using FASTQC v0.11.9 (Khairi et al., 2022), quality-filtered using Trimmomatic v0.36 and *de novo* assembled using SPAdes v3.7 (Vignolle et al., 2021). The assembled genomes were assessed for quality using QUAST v5.2.2 (Carneiro et al., 2023) and for completeness using BUSCO v5.3.2 (Khairi et al., 2022). The assembled genomes of C2HIR and C1HIR isolates were used for further comparative analysis.

3.2.17.2 Genome annotation and analysis

Augustus v.3.4.0 (Raman et al., 2021), a pipeline for ab-initio gene prediction was used to determine the putative genes present in the genomes of C2HIR and C1HIR. The predicted genes were translated into amino acid sequences and functionally annotated using EggNOG v5.0 (Huerta-Cepas et al., 2017), a database for ortholog relationships, functional annotation, and gene evolutionary histories. Later, genes responsible for virulence and pathogenicity were identified using Pathogen Host Interactions (PHI)-base (http://www.phi-base.org/) (Khairi et al., 2022), a database containing meticulously curated molecular and biological information about genes that have been verified to influence the outcomes of interactions between pathogens and their hosts.

3.2.17.3 Phylogenetic analysis of *Foc* isolates

Phylogeny was inferred using Maximum Likelihood method. The nuclear ribosomal Internal Transcribed Spacer (ITS) region sequences of 31 *Fusarium* isolates were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) using the accession numbers listed in Appendix E. All these ITS sequences, including those from C2HIR and C1HIR isolates were aligned using CLUSTAL-W method, and MEGA11 was used to construct the consensus phylogenetic tree using maximum likelihood algorithm and Kimura-2 parameter model with 1000 bootstrap replications. *Ilyonectria radicicola* served as the outgroup for this analysis.

3.2.17.4 Molecular characterization of *SIX* genes from Malaysian *Foc* isolates (C2HIR and C1HIR)

The presence or absence of *Secreted in Xylem* (*SIX*) genes in the Malaysian C2HIR and C1HIR isolates were analysed in the annotated genomes. A set of *SIX* gene database was created locally using all the *SIX* gene sequences obtained from National Centre for Biotechnology Information (NCBI) database and the assembled genomes were BLASTed against the database to identify the presence of the desired genes. The gene structures of the *SIX* genes were assessed by analysing number of introns and exons present in each gene. The sequence similarity of the *SIX* genes in the Malaysian C2HIR and C1HIR isolates were compared to the other published *Foc SIX* gene sequences.

CHAPTER 4: RESULTS

4.1 Morphological observation of *Foc*1_C2HIR and *Foc*4_C1HIR

Starter cultures from the glycerol stock were successfully cultured on full strength Difco™ Potato Dextrose Agar (PDA) supplemented with 500µl of streptomycin. White purplish mycelium was observed on PDA after incubation in a dark room for seven days at 25±2°C indicating germinated spores of *Foc*1 and *Foc*4 (Figure 4.1). The germinated fungal mycelia were inoculated in Potato Dextrose Broth (PDB) and incubated for another seven days until the broth (suspension culture) turned turbid which shows significant mycelium development (Figure 4.2). The suspension culture concentration was measured and diluted to a final concentration of $1x10^6$ spores/ml. This validated the viability of fungal suspension that was utilized for the bioassay experiment.

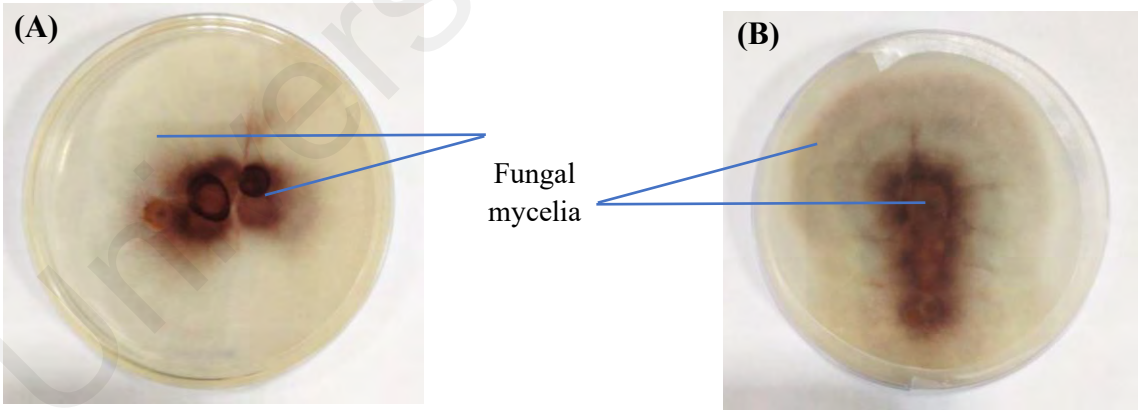


Figure 4.1: White purplish mycelium growth on Potato Dextrose Agar (PDA) after incubation for seven days in a dark room at 25±2°C. (A) *Foc*1 culture (B) *Foc*4 culture.

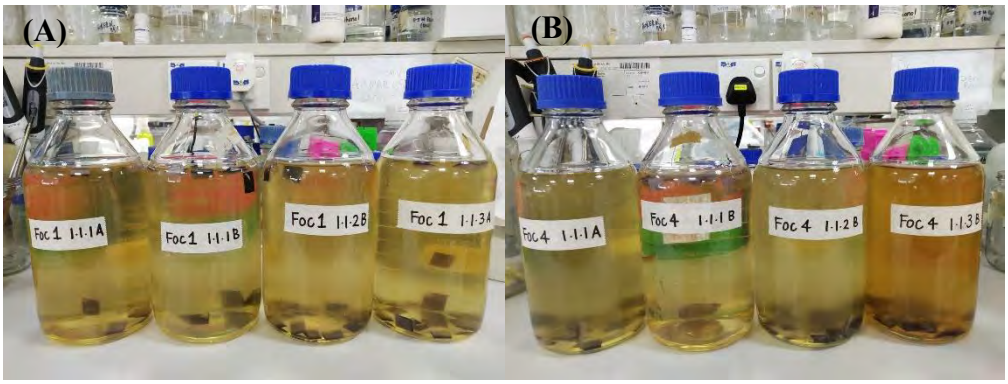


Figure 4.2: The *Foc* spore suspension cultures/inoculums that have turned turbid after incubation for seven days in a dark room at 25±2°C. (A) *Foc*1 suspension culture (B) *Foc*4 suspension culture.

4.2 DNA quantification and qualification of extracted *Foc* samples

The DNA was isolated from the fungal mycelia (Figure 4.1) and the concentration of extracted fungal DNA was measured using Nanodrop 2000 Spectrophotometer (Thermo Scientific). The concentration and A260/280 value obtained were (0.357µg/µl; 1.9, respectively) for *Foc*1 and (0.134µg/µl; 1.9, respectively) for *Foc*4 (Table 4.1).

Table 4.1: DNA concentration and purity of the extracted fungal samples

Samples	Concentration (µg/µl)	A260/280	A260/230
*Foc*1_C2HIR	0.357	1.900	1.293
*Foc*4_C1HIR	0.134	1.900	1.107

The DNA integrity was measured by gel electrophoresis in 1% agarose. The DNA bands obtained for both samples were intact and of good purity (Figure 4.3).

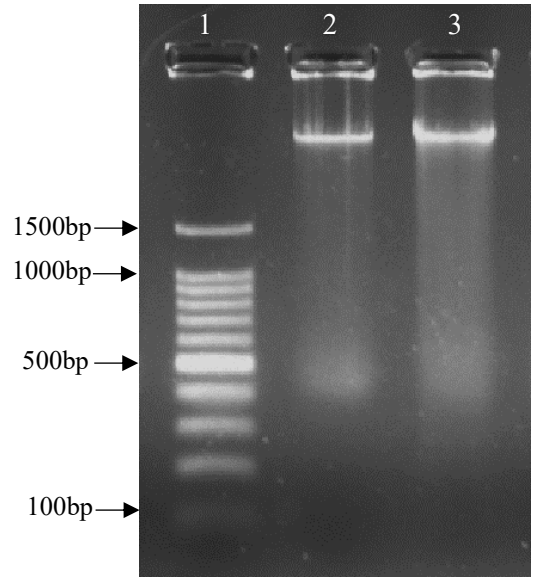


Figure 4.3: Agarose gel electrophoresis of genomic DNA from *Foc*1_C2HIR and *Foc*4_C1HIR. Lane 1: 100bp ladder; Lane 2: *Foc*1 DNA; Lane 3: *Foc*4 DNA.

4.3 PCR amplification for detection of *Foc*1_C2HIR and *Foc*4_C1HIR using race-specific primers

Prior to Fusarium wilt bioassay, DNA extraction was carried out from *Foc*1 and *Foc*4 fungal cultures (section 4.1) to confirm the isolates. The concentration and purity of the extracted DNA were as mentioned previously in section 4.2. These *Foc*1 and *Foc*4 DNA samples were then amplified with race-specific primers. A single bright band was observed for each race with amplicon size of *Foc*1 (245bp) and *Foc*4 (242bp). The presence of expected amplicon size has confirmed the samples as *Foc*1 and *Foc*4 respectively (Figure 4.4).

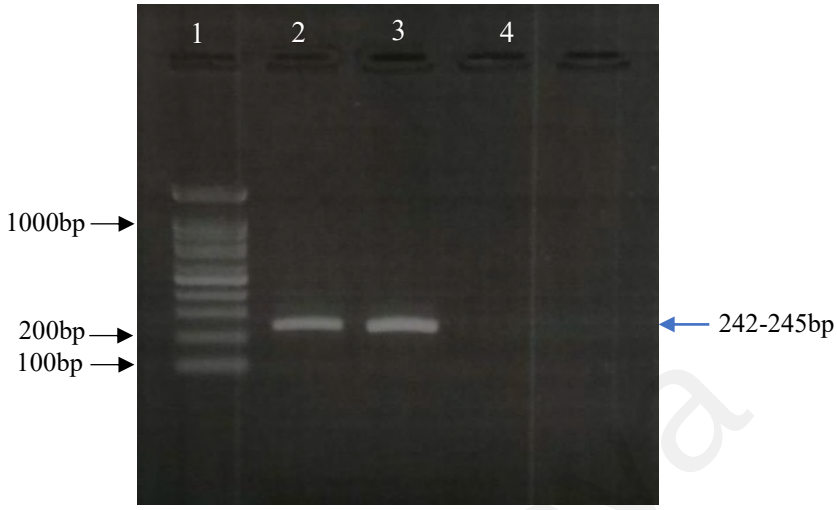


Figure 4.4: PCR amplification of *Foc*1 and *Foc*4 DNA with race-specific primers. Lane 1: 100bp ladder, Lane 2: *Foc*1 (245bp), Lane 3: *Foc*4 (242bp), Lane 4: negative control (without DNA).

4.4 Pathogenicity test for *Foc*-infected cv. Berangan plants

The Fusarium wilt bioassay method was utilised in this study to determine the pathogenicity level of the *Foc*1 and *Foc*4 isolates. A visual evaluation of external symptoms of Fusarium wilt was conducted on 12 cv. 'Berangan' plants at the end of 5th week post-inoculation for confirmation of disease progression. The *Foc*4-infected plants showed more severe yellowing symptoms compared to *Foc*1-infected plants. Internally, the rhizome of the *Foc*4-infected plants displayed more discoloration compared to *Foc*1-infected plants. The plants treated with sterile distilled water remained healthy throughout the bioassay experiment. Figure 4.5 shows the cross section of banana plantlets treated with different inoculums.

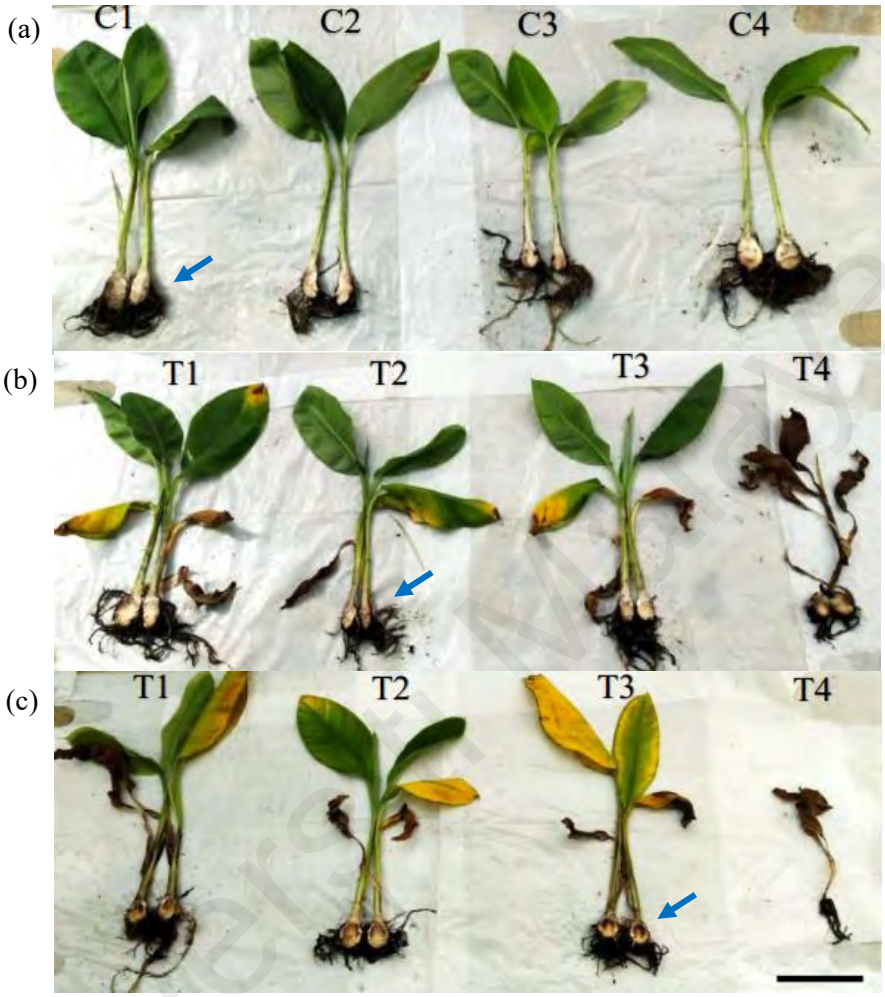


Figure 4.5: Cross section of banana plantlets treated with (a) Sterile distilled water (Control) (b) *Foc*1 (c) *Foc*4 at the end of 5th week post-inoculation. Bar represents 8cm.

The LSI and RDI scores, documented in Table 4.2, reflect the post-inoculation outcomes after 5 weeks. Remarkably, the control group plants consistently maintained LSI and RDI scores at an optimum level of 1. Conversely, plants subjected to *Foc*1 treatment exhibited a noteworthy spectrum, with LSI scores ranging from 3 to 5 and corresponding RDI scores of 4 to 8. Intriguingly, *Foc*4-inoculated plants displayed an elevated LSI score, varying between 4 and 5, alongside robust RDI scores ranging from 7 to 8.

Table 4.2: LSI and RDI scores of cv. 'Berangan' plants after 5 weeks post-inoculation with sterile distilled water, *Foc*1 suspension culture and *Foc*4 suspension culture.

Treatment group	Plant tag	LSI score	RDI score
Control (Sterile Distilled Water)	C1	1	1
	C2	1	1
	C3	1	1
	C4	1	1
Treatment 2 (*Foc*1)	T1	3	4
	T2	2	4
	T3	3	4
	T4	5	8
Treatment 3 (*Foc*4)	T1	4	7
	T2	4	7
	T3	4	7
	T4	5	8

For the pathogenicity test, final disease translation was computed using Disease Severity Index (DSI) based on data derived from the LSI and RDI. The final disease translation is documented in Table 4.3 and Table 4.4.

Table 4.3: Final disease translation of cv. 'Berangan' plants after 5 weeks post-inoculation with *Foc*1 suspension culture.

Index	Score	No of seedling	DSI	Translation	Final translation
Leaf Symptom Index (LSI)	1	0	$=\frac{\Sigma(3x2)+(2x1)+(5x1)}{\Sigma(2+1+1)}$ $=\frac{6+2+5}{4}$ $= 3.25$	Highly susceptible	Highly susceptible
	2	0			
	3	3			
	4	0			
	5	1			
Rhizome Discoloration Index (RDI)	1	0	$=\frac{\Sigma(4x3)+(8x1)}{\Sigma(3+1)}$ $=\frac{12+8}{4}$ $= 5.0$	Susceptible	
	2	0			
	3	0			
	4	0			
	5	1			
	6	2			
	7	0			
	8	1			

Table 4.4: Final disease translation of cv. 'Berangan' plants after 5 weeks post-inoculation with *Foc*4 suspension culture.

Index	Score	No of seedling	DSI	Translation	Final translation
Leaf Symptom Index (LSI)	1	0	$= \frac{\Sigma(4x3)+(5x1)}{\Sigma(3+1)}$ $= \frac{12+5}{4}$ $= 4.25$	Highly susceptible	Highly susceptible
	2	0			
	3	0			
	4	3			
	5	1			
Rhizome Discoloration Index (RDI)	1	0	$= \frac{\Sigma(7x3)+(8x1)}{\Sigma(3+1)}$ $= \frac{21+8}{4}$ $= 7.25$	Highly susceptible	
	2	0			
	3	0			
	4	0			
	5	0			
	6	0			
	7	3			
	8	1			

4.5 Fungal recovery assay

The 2 weeks post-inoculation (2wpi) banana root and stem tissues were isolated on a PDA to identify the colonization of *Foc*1 and *Foc*4 in vascular tissues. All the tissue samples showed a positive fungal growth (purplish white mycelia) after five days of incubation at room temperature (Figure 4.6 – 4.9). The fungus was later confirmed as *Foc*1 and *Foc*4 respectively, after analyzing the sequencing results (Appendix B).

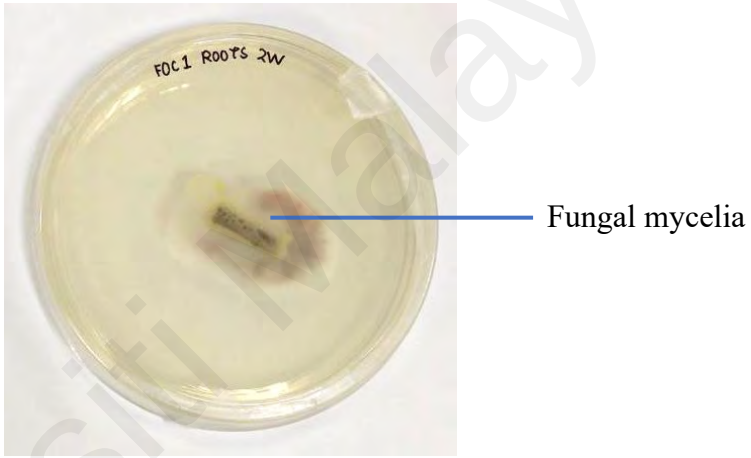


Figure 4.6: Fungal mycelia growth from the *Foc*1-infected root sample on PDA after five days of incubation at room temperature.

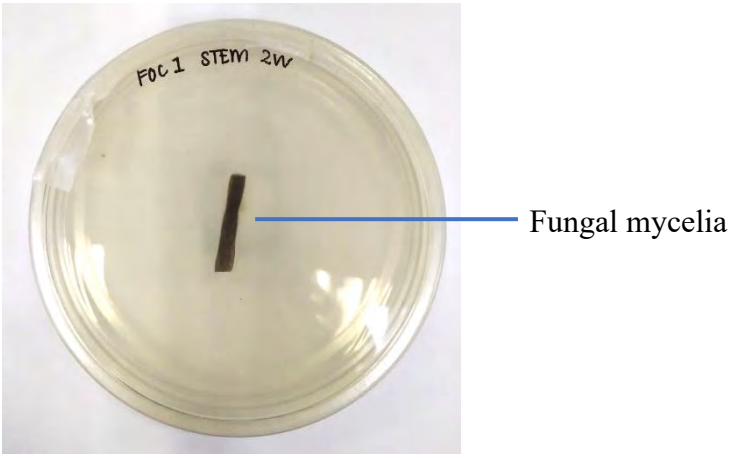


Figure 4.7: Fungal mycelia growth from the *Foc*1-infected stem sample on PDA after five days of incubation at room temperature.

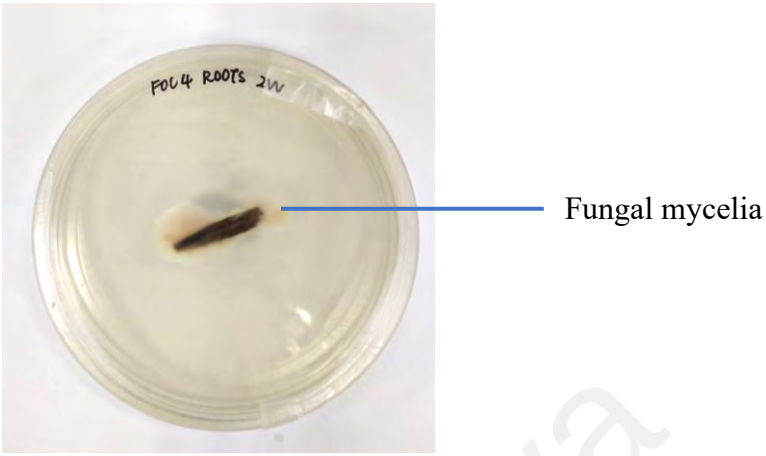


Figure 4.8: Fungal mycelia growth from the *Foc*4-infected root sample on PDA after five days of incubation at room temperature.

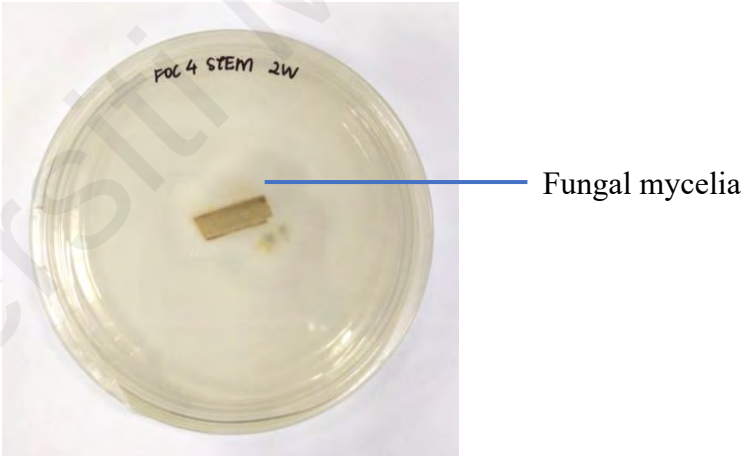


Figure 4.9: Fungal mycelia growth from the *Foc*4-infected stem sample on PDA after five days of incubation at room temperature.

4.6 PCR amplification for detection of *SIX* genes in *Foc* DNA

4.6.1 *SIX* genes in *Foc*1

*Foc*1 DNA sample was amplified with specific *SIX* gene primers. The gene-specific primers produced a distinct band with expected amplicon size for *SIX9b* (461bp) (Figure 4.10). *SIX1*, *SIX6*, *SIX8a* (Figure 4.10) as well as *SIX7* and *SIX13* were not amplified in this reaction. The amplified *SIX* gene PCR product was purified and sent for sequencing for validation and confirmation.

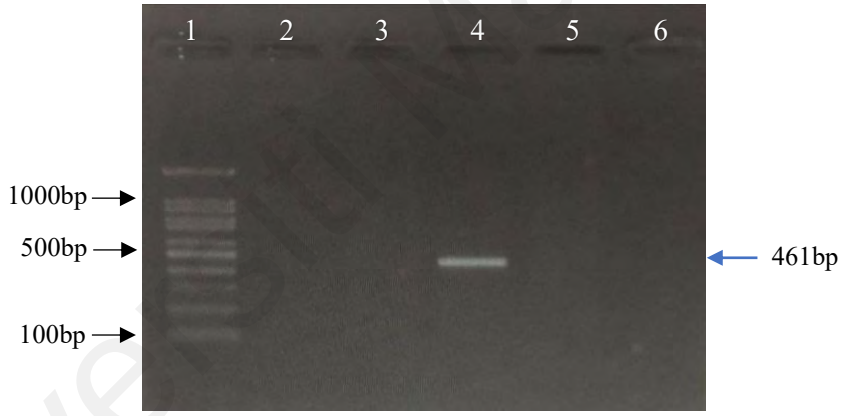


Figure 4.10: PCR amplification using gene specific primers for detection of *SIX1*, *SIX6, SIX9b* and *SIX8a* in *Foc*1 DNA. Lane 1: 100bp ladder; Lane 2: *SIX1*; Lane 3: *SIX6*; Lane 4: *SIX9b* (461bp); Lane 5: *SIX8a*; Lane 6: negative control (without DNA).

The *Foc*1 DNA was later amplified with qPCR primers to confirm the specificity of the designed primers. A single bright band was observed for *SIX9b* gene with amplicon size 122bp (Figure 4.11).

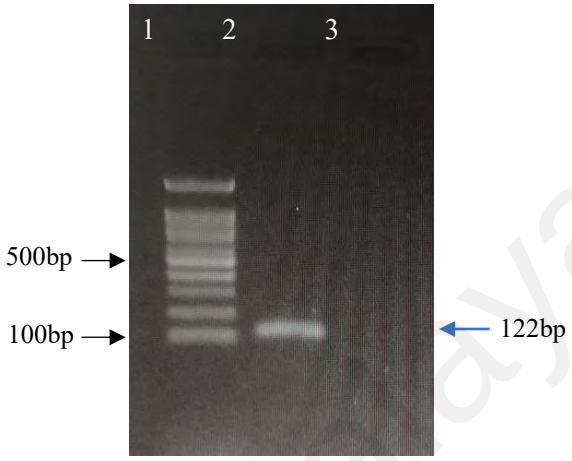


Figure 4.11: PCR amplification using gene-specific qPCR primers for detection of *SIX9b* in *Foc*1 DNA. Lane 1: 100bp ladder; Lane 2: *SIX9b* (122bp); Lane 3: negative control (without DNA).

4.6.2 Sequencing analysis for *SIX* genes in *Foc*1

The sequenced results were first analysed using BioEdit Sequence Alignment Editor software. The chromatogram of forward and reverse sequence for each gene was analysed to check for clear single peaks and the noises were trimmed. Later, a consensus sequence was created using both forward and reverse sequences for each gene. The consensus sequences were then blasted in NCBI database to confirm the specific gene product (highest homology with *Foc* strain BRIP63259). The sequencing analysis results for *SIX9b* gene are shown in Figure 4.12, 4.13, 4.14 and 4.15.

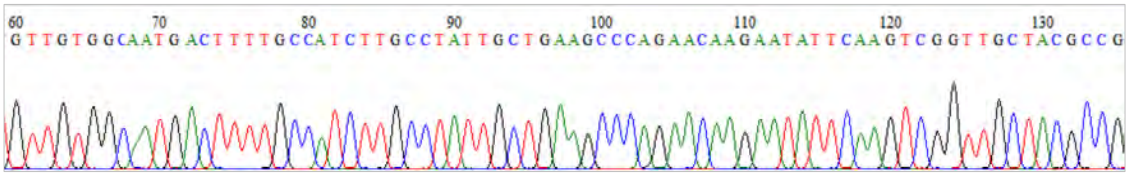


Figure 4.12: The chromatogram for forward sequence of *SIX9b* gene.

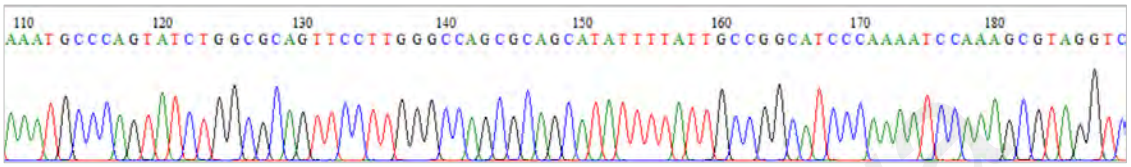
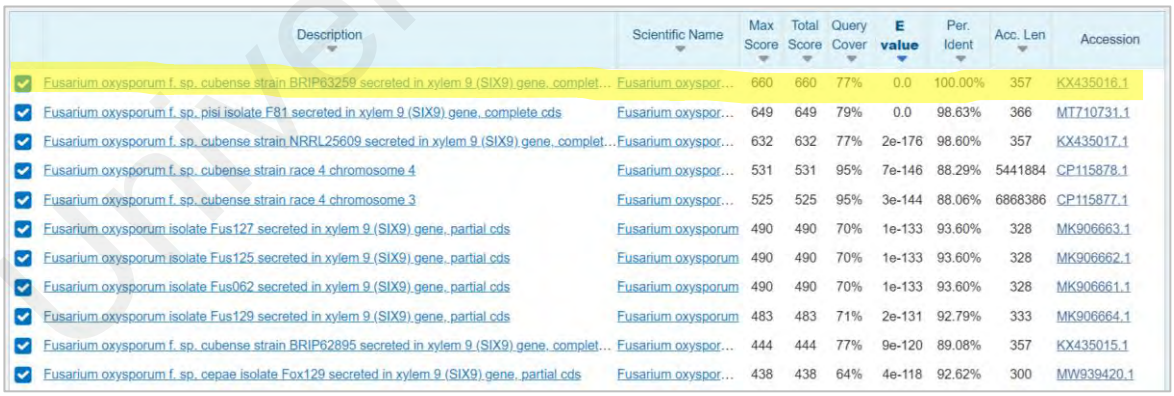


Figure 4.13: The chromatogram for reverse sequence of *SIX9b* gene.

Figure 4.14: The consensus sequence obtained from alignment of both forward and reverse sequence of *SIX9b* gene.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fusarium oxysporum f. sp. cubense strain BRIP63259 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxyspor...	660	660	77%	0.0	100.00%	357	KX435016.1
Fusarium oxysporum f. sp. pisi isolate F81 secreted in xylem 9 (SIX9) gene, complete cds	Fusarium oxyspor...	649	649	79%	0.0	98.63%	366	MT710731.1
Fusarium oxysporum f. sp. cubense strain NRRL25609 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxyspor...	632	632	77%	2e-176	98.60%	357	KX435017.1
Fusarium oxysporum f. sp. cubense strain race 4 chromosome 4	Fusarium oxyspor...	531	531	95%	7e-146	88.29%	5441884	CP115878.1
Fusarium oxysporum f. sp. cubense strain race 4 chromosome 3	Fusarium oxyspor...	525	525	95%	3e-144	88.06%	6868386	CP115877.1
Fusarium oxysporum isolate Fus127 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	490	490	70%	1e-133	93.60%	328	MK906663.1
Fusarium oxysporum isolate Fus125 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	490	490	70%	1e-133	93.60%	328	MK906662.1
Fusarium oxysporum isolate Fus062 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	490	490	70%	1e-133	93.60%	328	MK906661.1
Fusarium oxysporum isolate Fus129 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	483	483	71%	2e-131	92.79%	333	MK906664.1
Fusarium oxysporum f. sp. cubense strain BRIP62895 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxyspor...	444	444	77%	9e-120	89.08%	357	KX435015.1
Fusarium oxysporum f. sp. cepae isolate Fox129 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxyspor...	438	438	64%	4e-118	92.62%	300	MW939420.1

Figure 4.15: The BLAST result obtained from NCBI database has confirmed the sequence as *SIX9b* gene with 100% identity.

4.6.3 *SIX* genes in *Foc*4

*Foc*4 DNA sample was amplified with specific *SIX* gene primers. The gene specific primers produced a distinct band with expected amplicon size i.e., *SIX1* (923bp), *SIX2* (806-810bp), *SIX4* (930-937bp), *SIX6* (754bp), *SIX8a* (624bp), *SIX9a* (424bp), *SIX13* (1076-1082bp). The *SIX7* gene was not amplified in this reaction. The PCR amplification results are shown in Figure 4.16, 4.17 and 4.18.

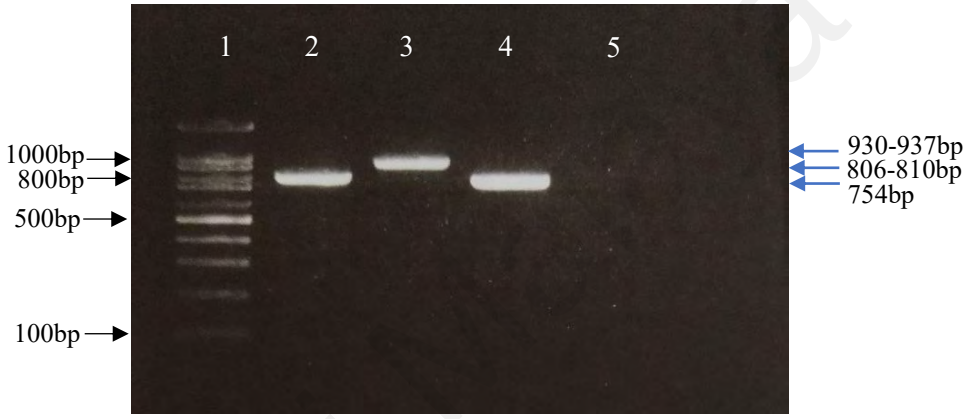


Figure 4.16: PCR amplification using gene-specific primers for detection of *SIX2*, *SIX4,* and *SIX6* in *Foc*4 DNA. Lane 1: 100bp ladder; Lane 2: *SIX2* (806-810bp); Lane 3: *SIX4* (930-937bp); Lane 4: *SIX6* (754bp); Lane 5: negative control (without DNA).

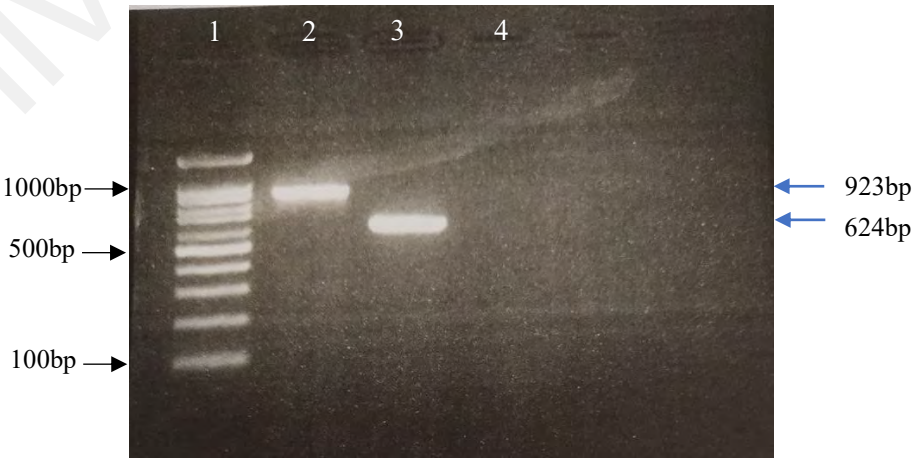


Figure 4.17: PCR amplification using gene-specific primers for detection of *SIX1*, and *SIX8a* in *Foc*4 DNA. Lane 1: 100bp ladder; Lane 2: *SIX1* (923bp); Lane 3: *SIX8a* (624bp); Lane 4: negative control (without DNA).

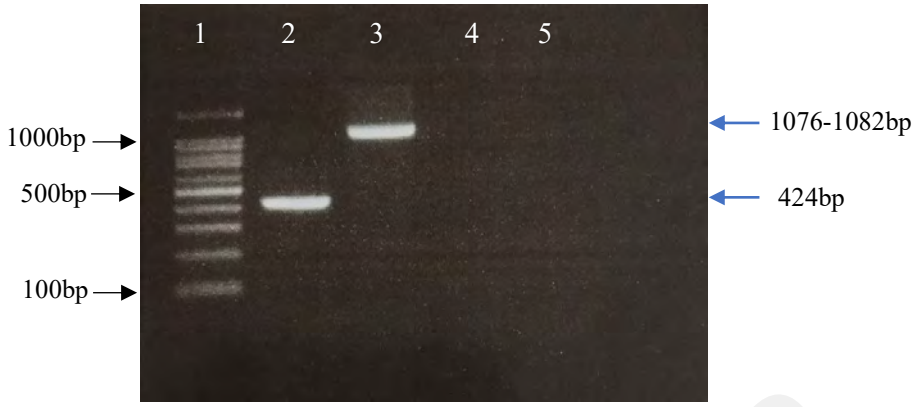


Figure 4.18: PCR amplification using gene-specific primers for detection of *SIX9a*, *SIX13,* and *SIX7* in *Foc*4 DNA. Lane 1: 100bp ladder; Lane 2: *SIX9a* (424bp); Lane 3: *SIX13* (1076-1082bp); Lane 4: *SIX7*; Lane 5: negative control (without DNA).

All the amplified *SIX* gene PCR products were purified and sent for sequencing for validation and confirmation.

The *Foc*4 DNA was later amplified with qPCR primers to confirm the specificity of the designed primers. A single bright band was observed for each gene with amplicon size of *SIX1* (119bp), *SIX6* (90bp), *SIX8a* (96bp), *SIX9a* (111bp) as shown in Figure 4.19.

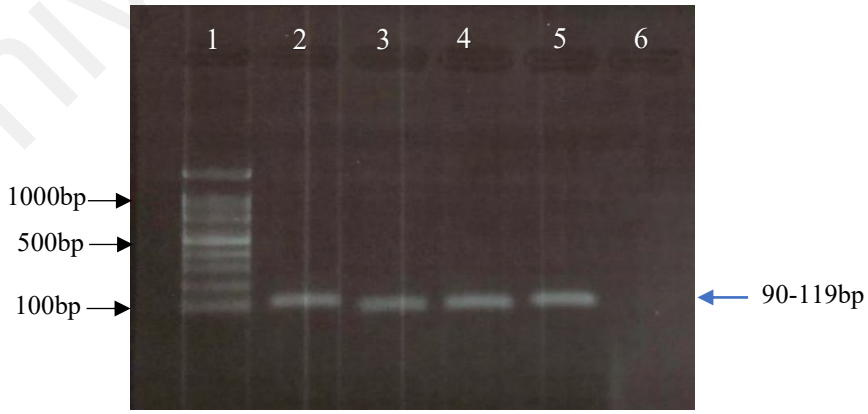


Figure 4.19: PCR amplification using gene-specific qPCR primers for detection of *SIX1*, *SIX6, SIX8a* and *SIX9a* in *Foc*4 DNA. Lane 1: 100bp ladder; Lane 2: *SIX1* (119bp); Lane 3: *SIX6* (90bp); Lane 4: *SIX8a* (96bp); Lane 5: *SIX9a* (111bp); Lane 6: negative control (without DNA).

4.6.4 Sequencing analysis for *SIX* genes in *Foc*4

The sequenced results were first analysed using BioEdit Sequence Alignment Editor software. The chromatogram of forward and reverse sequence for each gene was analysed to check for clear single peaks and the noises were trimmed. Later, a consensus sequence was created using both forward and reverse sequences for each gene. The consensus sequences were then blasted in NCBI database to confirm the specific gene product. The sequencing analysis results for *SIX9a* gene (representative results) are shown in Figure 4.20, 4.21, 4.22 and 4.23. The sequencing results for other *SIX* genes are attached in Appendix B.

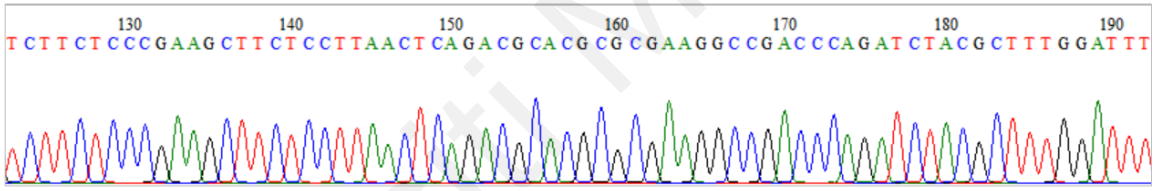


Figure 4.20: The chromatogram for forward sequence of *SIX9a* gene.

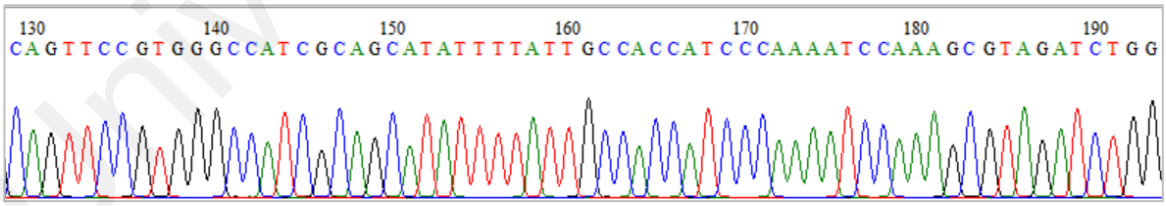


Figure 4.21: The chromatogram for reverse sequence of *SIX9a* gene.

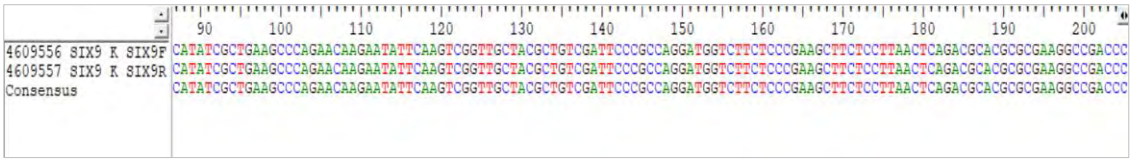


Figure 4.22: The consensus sequence obtained from alignment of both forward and reverse sequence of *SIX9a* gene.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fusarium oxysporum f. sp. cubense strain BRIP62895 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxysporum f....	660	660	90%	0.0	100.00%	357	KX435015.1
Fusarium odoratissimum NRRL 54006 uncharacterized protein (FOIG_16496), partial mRNA	Fusarium odoratissimu...	616	616	84%	1e-171	100.00%	333	XM_031217957.1
Fusarium oxysporum isolate Fus127 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	466	466	78%	1e-126	93.61%	328	MK906663.1
Fusarium oxysporum isolate Fus125 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	466	466	78%	1e-126	93.61%	328	MK906662.1
Fusarium oxysporum isolate Fus062 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	466	466	78%	1e-126	93.61%	328	MK906661.1
Fusarium oxysporum isolate Fus129 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum	457	457	77%	7e-124	93.51%	333	MK906664.1
Fusarium oxysporum f. sp. cubense strain BRIP63259 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxysporum f....	444	444	90%	6e-120	89.08%	357	KX435016.1
Fusarium oxysporum f. sp. pisi isolate F81 secreted in xylem 9 (SIX9) gene, complete cds	Fusarium oxysporum f....	444	444	92%	6e-120	88.52%	366	MT710731.1
Fusarium oxysporum f. sp. cepae isolate Fox129 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum f....	442	442	75%	2e-119	92.95%	300	MW939420.1
Fusarium oxysporum f. sp. cepae isolate Fox220a secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum f....	442	442	75%	2e-119	92.95%	300	MW939414.1
Fusarium oxysporum f. sp. cepae isolate Fox260 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum f....	442	442	75%	2e-119	92.95%	300	MW939406.1
Fusarium oxysporum f. sp. narcissi isolate FOXN7 secreted in xylem 9 (SIX9) gene, partial cds	Fusarium oxysporum f....	429	429	75%	2e-115	92.64%	298	KP964979.1
Fusarium oxysporum f. sp. cubense strain NRRL25609 secreted in xylem 9 (SIX9) gene, complet...	Fusarium oxysporum f....	427	427	90%	6e-115	88.24%	357	KX435017.1

Figure 4.23: The BLAST result obtained from NCBI database has confirmed the sequence as *SIX9a* gene with 100% identity.

4.7 Real-time PCR (qPCR)

4.7.1 RNA quality and integrity

RNA was isolated from the *Foc*-infected banana root samples on the specific time points (0 days, 2 weeks, 4 weeks and 5 weeks). Gel electrophoresis was conducted to check the RNA integrity (Figure 4.24). All the samples showed typical band pattern of 28S and 18S. The total RNA purity of A260/280 was in the range of 1.7 – 2.0 indicating a good purity. The genomic DNA was removed by DNase treatment before proceeding with cDNA synthesis.

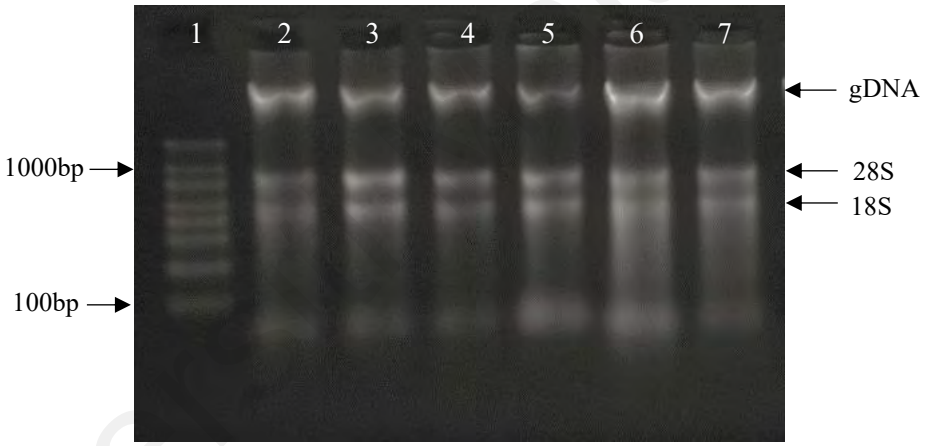


Figure 4.24: Representative gel image of RNA bands obtained from *Foc*-infected banana root samples. Lane 1: 100bp ladder; Lane 2-Lane 7: RNA samples

4.7.2 Amplification of cDNA with endogenous gene

The synthesized cDNAs from fungal RNA of infected banana root samples were amplified with an endogenous gene, *TEF1α*, to confirm a successful conversion of RNA to cDNA. A bright band was observed for each sample being amplified with expected band size of 120bp. Figure 4.25 shows a representative gel image for the samples tested.

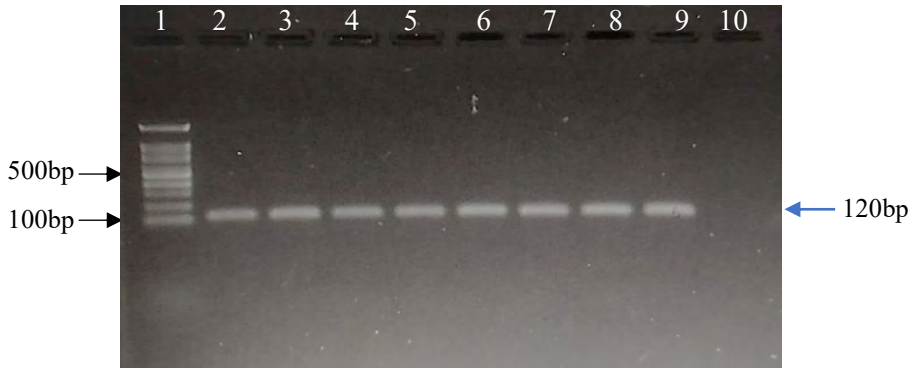


Figure 4.25: PCR amplification using endogenous gene, *TEF1α*, for validation of cDNA samples. Lane 1: 100bp ladder; Lane 2 – Lane 9: cDNA samples (120bp); Lane 10: negative control (without DNA)

4.7.3 Validation of reference genes

Based on a few literature reports, three housekeeping genes, *TEF1α, TUB* and *GAPDH* were selected as candidate reference genes. The primer efficiency test was conducted to validate the specificity and efficiency of the primers over a few concentrations. A single peak melt curve was obtained for *TEF1α* (Figure 4.26) and *TUB* (Figure 4.27) while double peaks formed for *GAPDH* (Figure 4.28).

A standard curve comprising five serial two-fold dilutions of cDNA was employed to ascertain the qPCR efficiency of each set of primers used. All reactions displayed a correlation coefficient of $R^2 > 0.98$ and efficiencies, $E > 1.8$. The results, Ct value or cycle threshold, were plotted against the ln of each dilution factor. The PCR efficiency obtained for *TEF1α* (1.99) and *TUB* (2.07) indicated a good primer specificity and amplification efficiency whereas *GAPDH* (6.71) indicating an inaccurate primer efficiency.

Since *GAPDH* did not show good efficiency, *TEF1a* and *TUB* were shortlisted as the candidate reference genes. Although these two genes were taken from previously published papers, however, an additional stability test was performed using Delta CT method to determine if they are stable under different treatment conditions over all the

time points studied. The genes with the lowest standard deviation (SD) value were regarded as the most stable genes. In our study, the standard deviation of both *TEF1a* and *TUB* were both in the range of ≤ 1.0 (Appendix D). Following the analysis, *TEF1α* and *TUB* were selected as the most suitable reference genes to be used in this gene expression study as they showed good efficiency, specificity and stability.

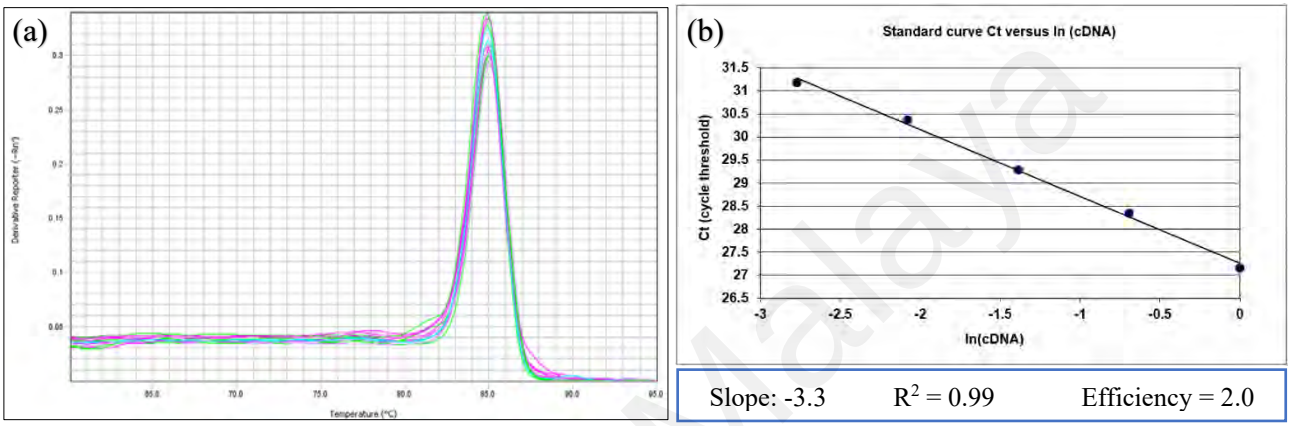


Figure 4.26: The (a) melt curve and (b) standard curve obtained for *TEF1α* primer efficiency test.

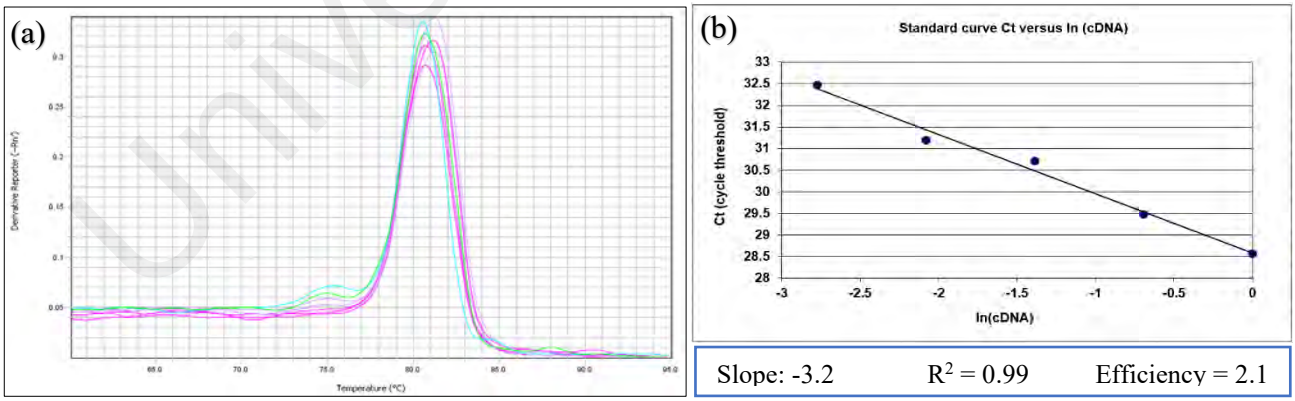


Figure 4.27: The (a) melt curve and (b) standard curve obtained for *TUB* primer efficiency test.

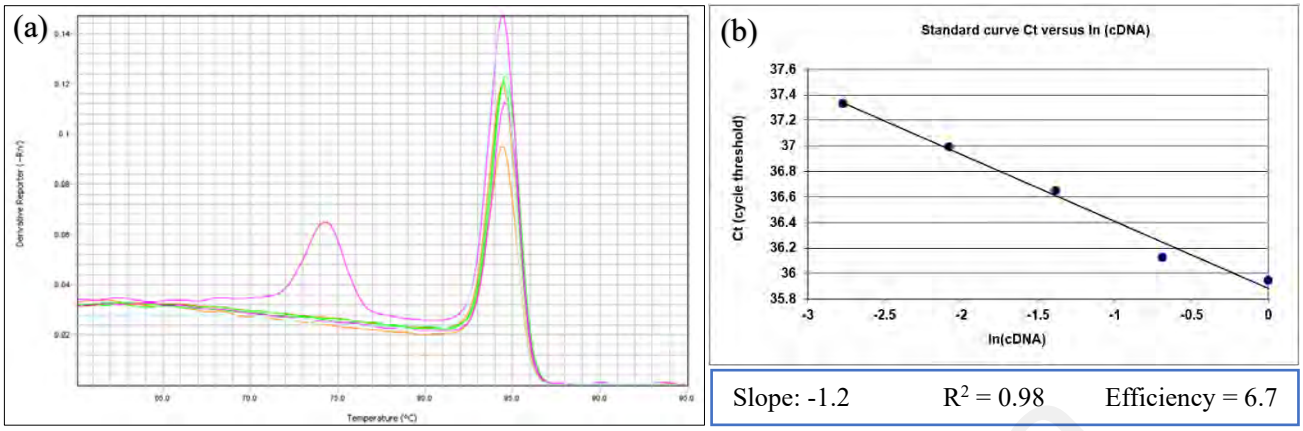


Figure 4.28: The (a) melt curve and (b) standard curve obtained for *GAPDH* primer efficiency test.

4.7.4 Validation of target genes

A target gene (*SIX9b*) was utilized for gene expression study in *Foc*1-infected banana plants while four target genes (*SIX1, SIX6, SIX8a, SIX9a*) were shortlisted for gene expression studies in *Foc*4-infected banana plants. The primer efficiency test was conducted to validate the specificity and efficiency of the primers over a few concentrations. A single peak melt curve was obtained for all the target genes (Figure 4.29 – 4.33). All the target genes were analyzed as suitable to be used in this expression study.

A standard curve of five serial two-fold dilutions of cDNA was used to determine the qPCR efficiency of each primer sets used. All reactions displayed a correlation coefficient of $R^2 > 0.98$ and efficiencies, $E > 1.8$. The results, Ct value or cycle threshold, were plotted against the ln of each dilution factor. The PCR efficiency obtained for *SIX9b* (2.1), *SIX1* (1.9), *SIX6* (1.9), *SIX8a* (1.9) and *SIX9a* (1.9) indicated good primer specificity and amplification efficiency.

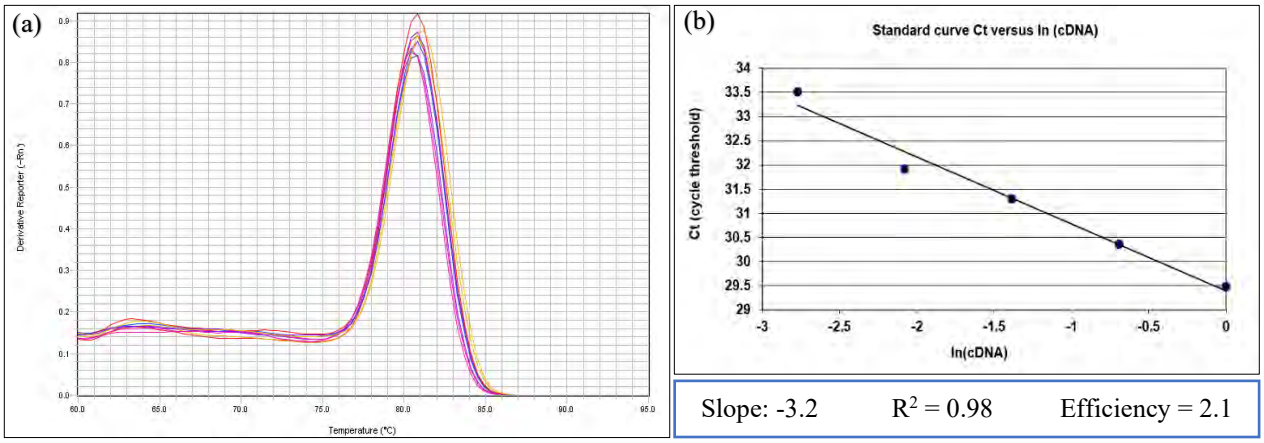


Figure 4.29: The (a) melt curve and (b) standard curve obtained for *SIX9b* primer efficiency test.

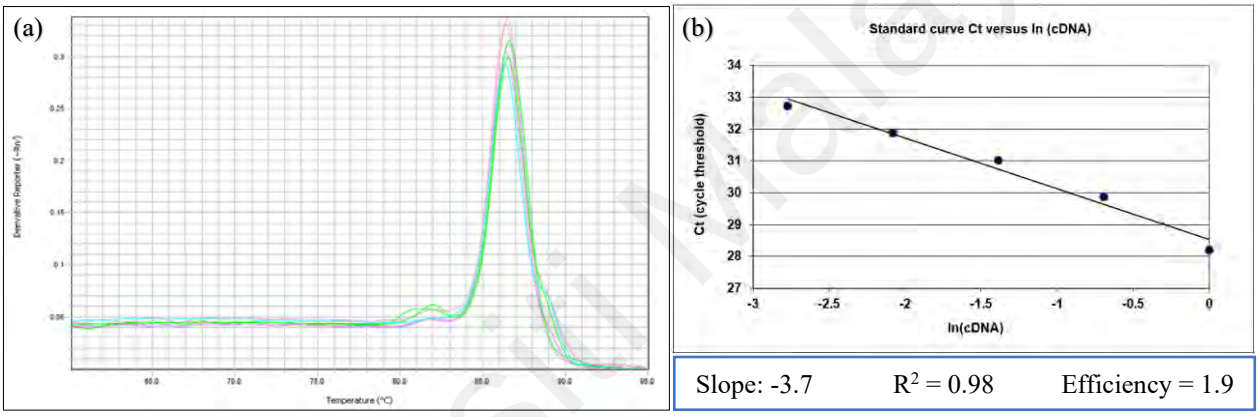


Figure 4.30: The (a) melt curve and (b) standard curve obtained for *SIX1* primer efficiency test.

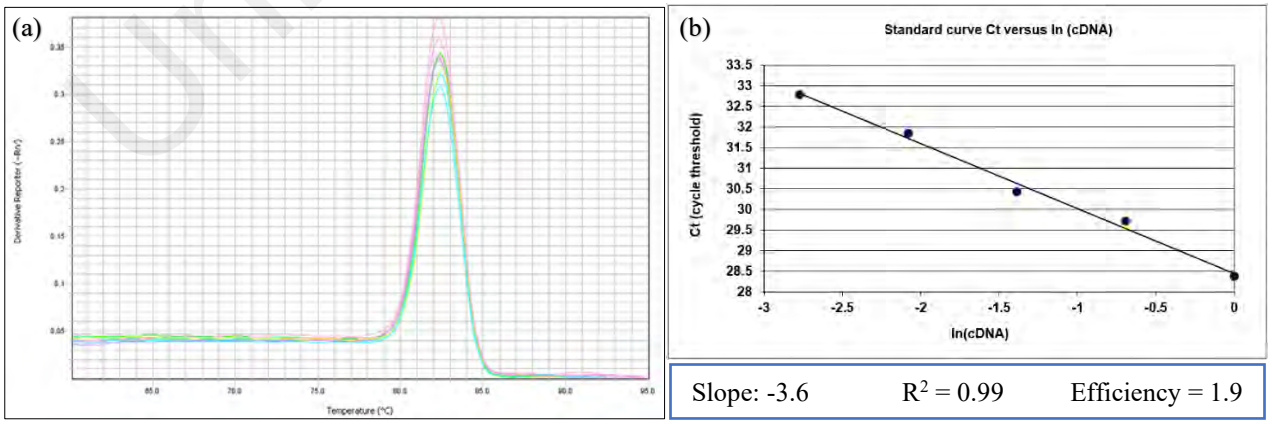


Figure 4.31: The (a) melt curve and (b) standard curve obtained for *SIX6* primer efficiency test.

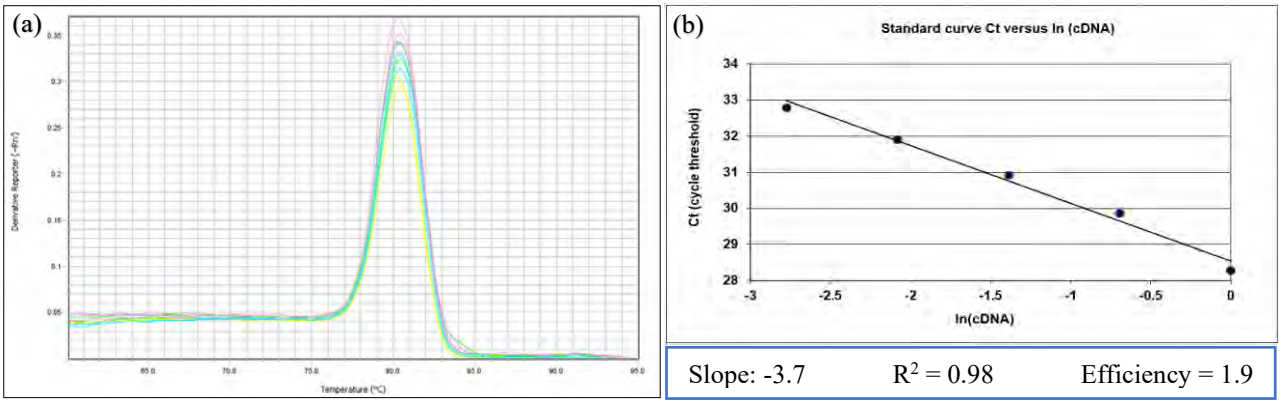


Figure 4.32: The (a) melt curve and (b) standard curve obtained for *SIX8a* primer efficiency test.

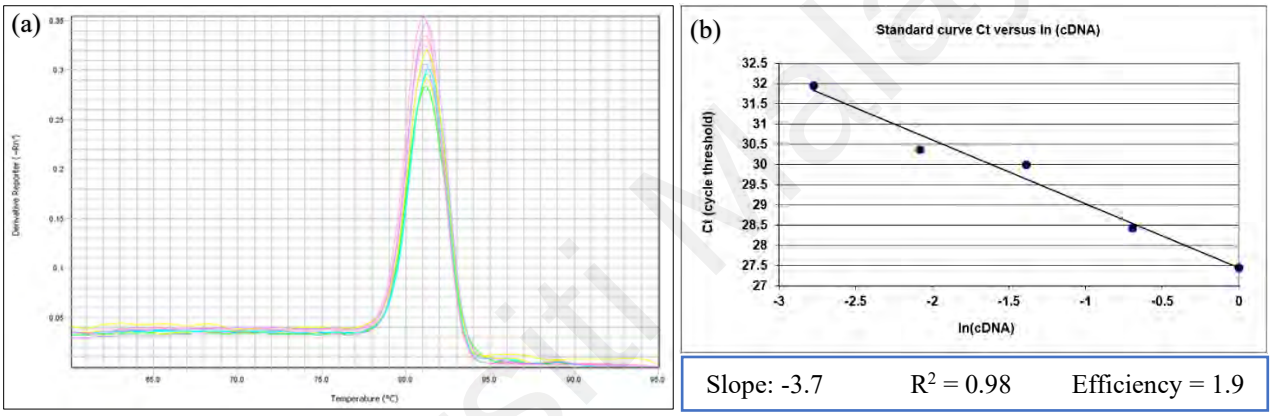


Figure 4.33: The (a) melt curve and (b) standard curve obtained for *SIX9a* primer efficiency test.

4.7.5 Gene expression studies of *SIX* genes in *Foc*1

The cDNA of *Foc*1 from infected banana root samples at selected time points i.e., 0 day, 2 weeks, 4 weeks and 5 weeks was used as template for this expression study. The *SIX* gene expression was studied across these time points. The *Foc*1-inoculated 0-day banana plants served as the control group. Differentially expressed *SIX* genes were detected in this study. The fold changes were determined using $2^{-\Delta\Delta CT}$ method and the results represent mean standard deviation (± SD) values of the samples studied.

4.7.5.1 *Secreted in Xylem* (*SIX9b*)

The qPCR results revealed that *SIX9b* was downregulated in the 2nd and 4th week with 0.80- and 0.65-fold change respectively whereas significantly upregulated with 5.22-fold changes on 5th week post-inoculation (Figure 4.34).

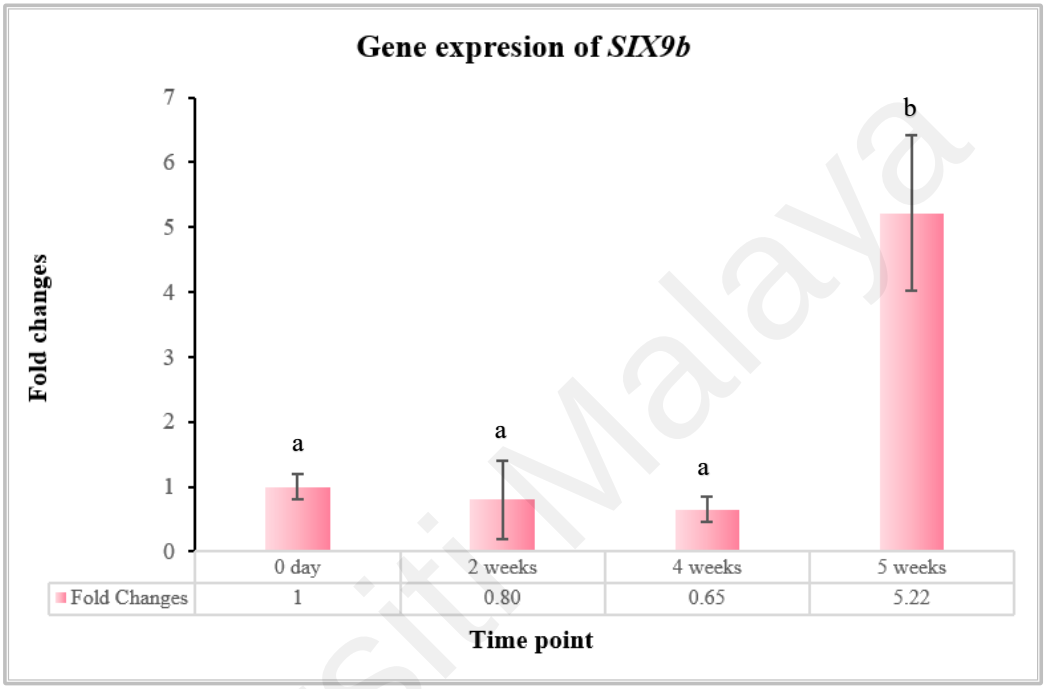


Figure 4.34: The fold changes in the expression of *SIX9b* gene in cv. 'Berangan' plants infected by *Fusarium oxysporum* f.sp. *cubense* Race 1 (*Foc*1) at different time points (2 weeks, 4 weeks, 5 weeks). 0-day plants served as the control for this experiment. The error bar represents the standard deviation of the results obtained. Different letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test p<0.05).

4.7.6 Gene expression studies of *SIX* genes in *Foc*4

The cDNA of *Foc*4 from infected banana root samples at selected time points i.e., 0 day, 2 weeks, 4 weeks and 5 weeks was used as template for this expression study. The *SIX* gene expression was studied across these time points. The *Foc*4-inoculated 0-day banana plants served as the control group. Differentially expressed *SIX* genes were detected in this study. The fold changes were determined using $2^{-\Delta\Delta CT}$ method and the

results represent mean standard deviation (± SD) values of the samples studied. The relative expression data was normalized to the average of *TEF1α* and *TUB,* ensuring a standardized and accurate representation. This enhances the reliability of the data for meaningful comparisons across different samples and conditions.

4.7.6.1 *Secreted in Xylem* (*SIX1*)

The qPCR results revealed that *SIX1* was significantly upregulated in the 2nd and 5th week with 25.07- and 6.68-fold change respectively, while only a slight upregulation (2.61) was observed on 4th week post-inoculation (Figure 4.35).

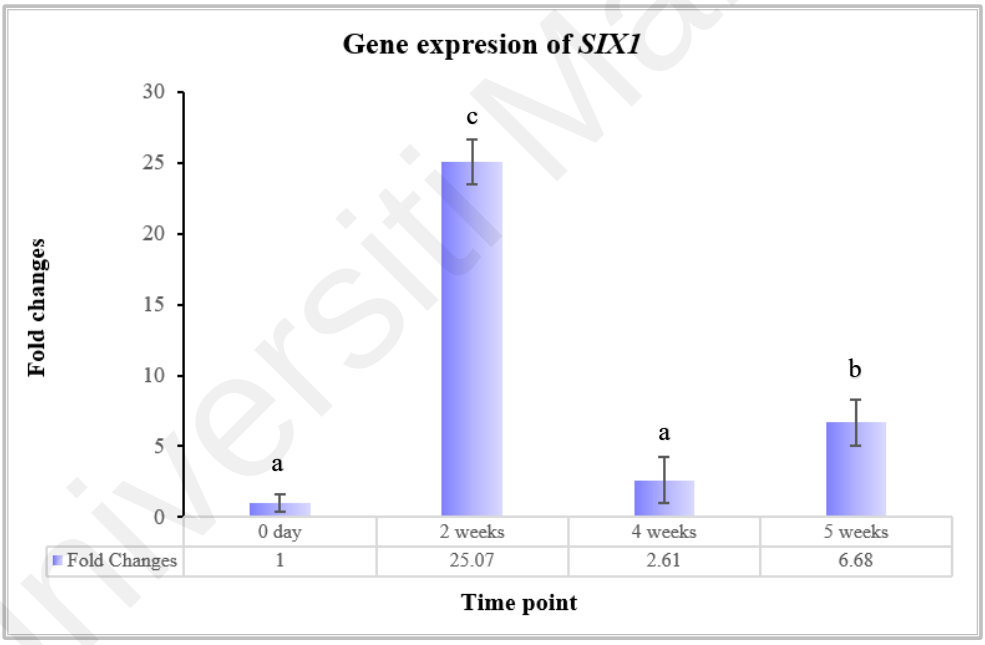


Figure 4.35: The fold changes in the expression of *SIX1* gene in cv. 'Berangan' plants infected by *Fusarium oxysporum* f.sp. *cubense* Race 4 (*Foc*4) at different time points (2 weeks, 4 weeks, 5 weeks). 0-day plants served as the control for this experiment. The error bar represents the standard deviation of the results obtained. Different letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test $p<0.05$).

4.7.6.2 *Secreted in Xylem* (*SIX6*)

The qPCR results revealed that *SIX6* was significantly upregulated in the 2nd week with 8.54-fold change. However, this gene was only slightly upregulated on 4th week (1.86) and 5th week (2.76) post-inoculation (Figure 4.36).

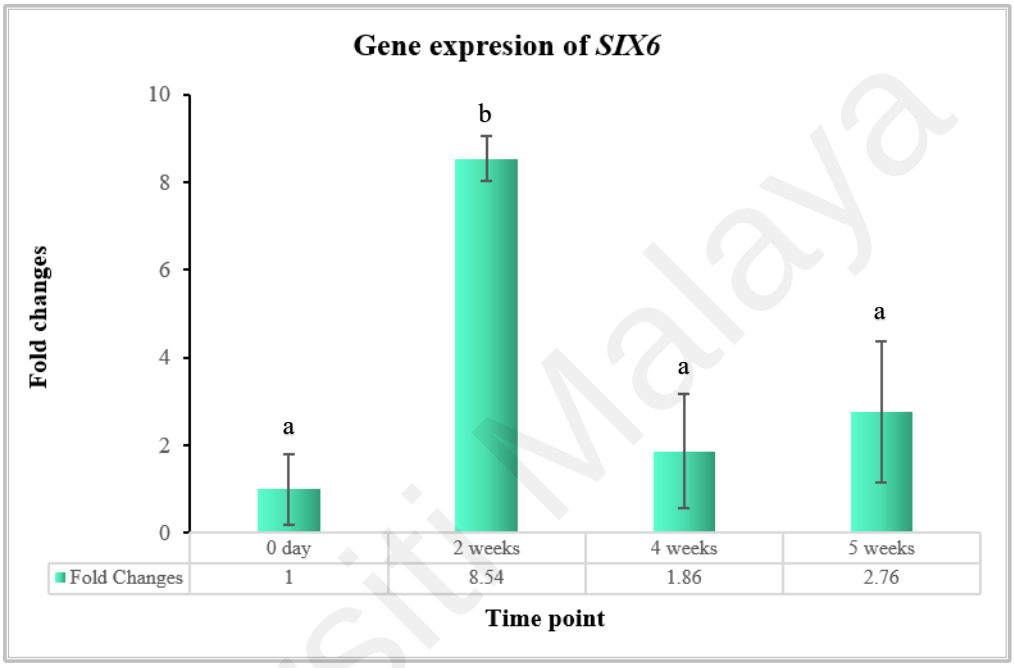


Figure 4.36: The fold changes in the expression of *SIX6* gene in cv. 'Berangan' plants infected by *Fusarium oxysporum* f.sp. *cubense* Race 4 (*Foc*4) at different time points (2 weeks, 4 weeks, 5 weeks). 0-day plants served as the control for this experiment. The error bar represents the standard deviation of the results obtained. Different letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test $p<0.05$).

4.7.6.3 *Secreted in Xylem* (*SIX8a*)

The qPCR results revealed that *SIX8a* was upregulated in the 2nd and 5th week with 6.31- and 4.92-fold change respectively, whereas a slight downregulation (0.88) in the expression was observed on 4th week post-inoculation (Figure 4.37).

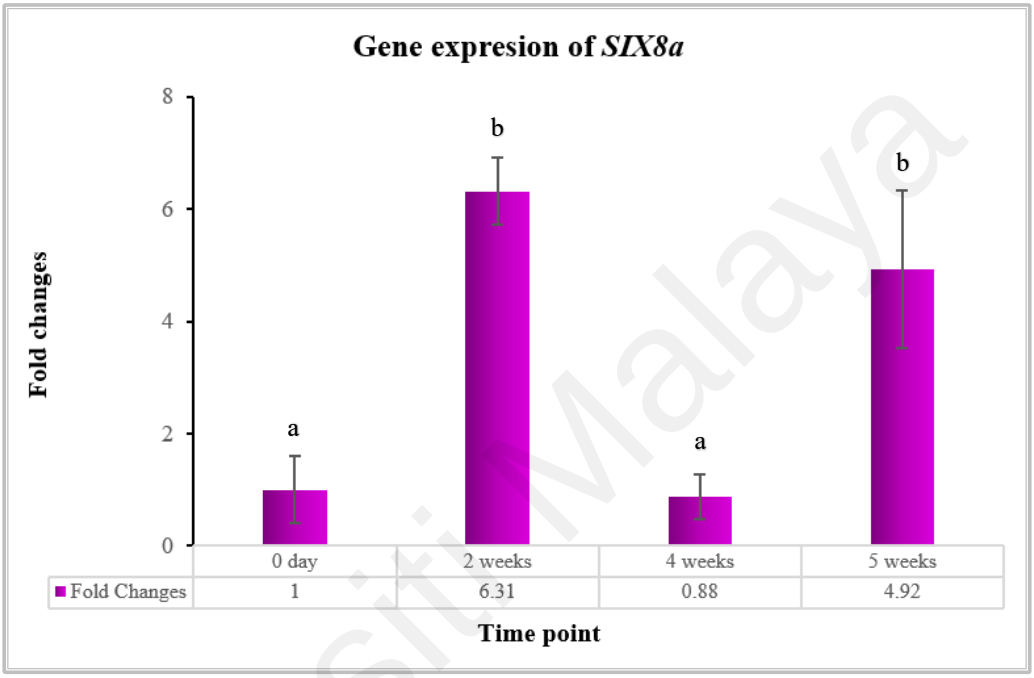


Figure 4.37: The fold changes in the expression of *SIX8a* gene in cv. 'Berangan' plants infected by *Fusarium oxysporum* f.sp. *cubense* Race 4 (*Foc*4) at different time points (2 weeks, 4 weeks, 5 weeks). 0-day plants served as the control for this experiment. The error bar represents the standard deviation of the results obtained. Different letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test $p<0.05$).

4.7.6.4 *Secreted in Xylem* (*SIX9a*)

The qPCR results revealed that *SIX9a* was significantly upregulated in the 2nd and 5th week with 12.17- and 7.04-fold change respectively, while it was slightly upregulated (1.91) on 4th week post-inoculation (Figure 4.38).

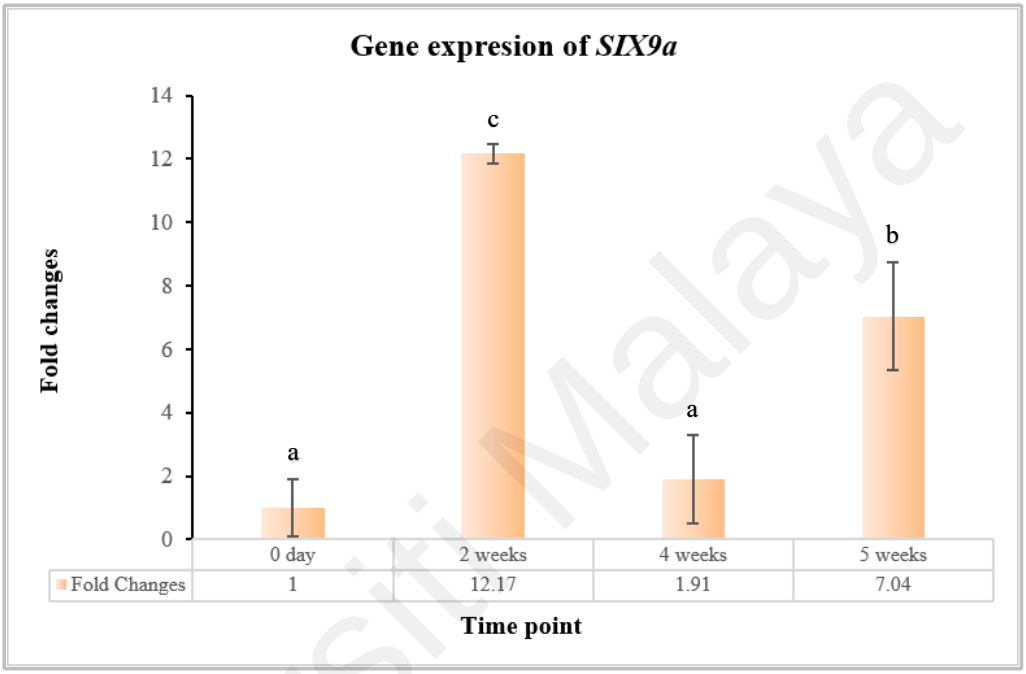


Figure 4.38: The fold changes in the expression of *SIX9a* gene in cv. 'Berangan' plants infected by *Fusarium oxysporum* f.sp. *cubense* Race 4 (*Foc*4) at different time points (2 weeks, 4 weeks, 5 weeks). 0-day plants served as the control for this experiment. The error bar represents the standard deviation of the results obtained. Different letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test $p<0.05$).

4.8 Whole genome sequencing and analysis

4.8.1 Quality assessment of the sequenced genomes

The raw sequence reads for *Foc*1_C2HIR and *Foc*4_C1HIR genomes obtained from the sequencer were assessed for overall quality using FASTQC and the average quality scores across all bases for both forward (R1) and reverse (R2) reads were shown in Figures 4.39 and 4.40. The average quality scores across all bases were indicated in Phred score (Q_{phred}) and they were all in the range of $Q_{phred}34$ to $Q_{phred}36$. This indicated that the raw sequence reads had achieved very good quality.

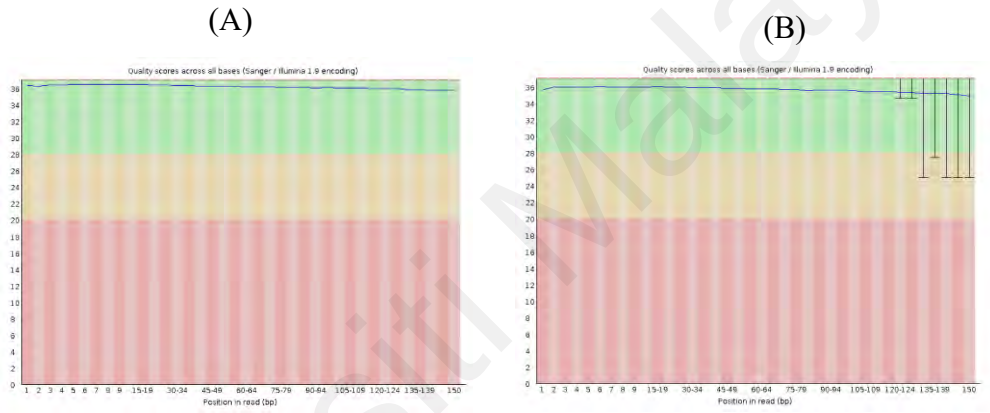


Figure 4.39: Average quality scores across all bases for the raw sequencing reads of *Foc*1_C2HIR genome. (A) Score for forward sequence reads (B) Score for reverse sequence reads.

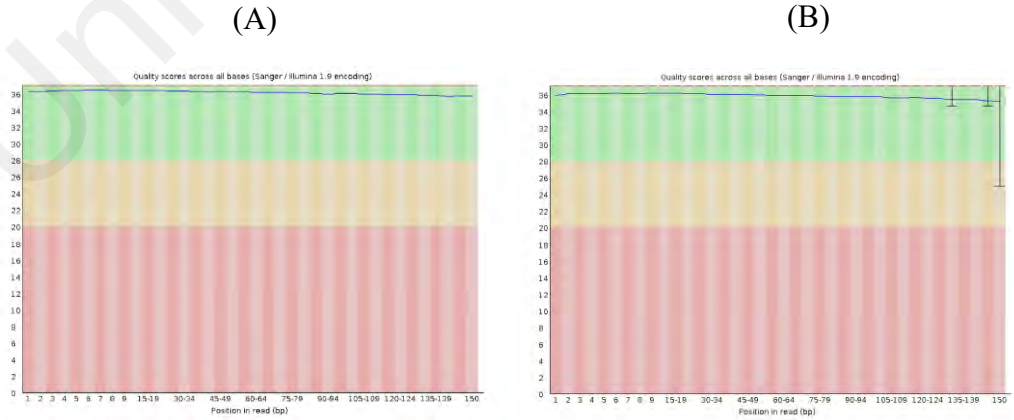


Figure 4.40: Average quality scores across all bases for the raw sequencing reads of *Foc*4_C1HIR genome. (A) Score for forward sequence reads (B) Score for reverse sequence reads.

4.8.2 Genomic feature analysis

The sequencing resulted in 4,116,443 and 5,107,922 raw reads for C2HIR and C1HIR, respectively. The quality-filtered reads were used for *de novo* assembly of C2HIR and C1HIR genomes. As a result, the assembled draft genome of C2HIR had a total length of 53,686,071bp with a 50.7% GC content and consisted of 1239 contigs. The BUSCO analysis revealed 97.3% genome completeness. Out of 4,494 BUSCOs, a total of 4370 BUSCOs were identified, 35 BUSCOs were fragmented and 89 were missing (Table 4.5). On the other hand, the assembled draft genome of C1HIR had a total length of 55,255,634bp with 50.5% GC content and consisted of 2824 contigs. The BUSCO analysis revealed 96.0% genome completeness. In this genome, a total of 4317 out of 4,494 BUSCOs were identified, 55 BUSCOs were fragmented and 122 were missing (Table 4.5). The genomic features of both C2HIR and C1HIR genomes were analyzed. Three reference genomes from a published study by Ma et al. (2010) and Raman et al. (2021) were included for a comparative analysis with the draft genome sequences obtained from our current study. A summary of the comparative analysis is tabulated in Table 4.5.

Table 4.5: A summary of genomic features of assembled genomes (C2HIR and C1HIR) and reference genomes.

Characteristics	*Foc*1 **C2HIR**	*Foc*4 **C1HIR**	*Reference 1* ***Fol*4287**	*Reference 2* **VCG01213/16 (*Foc*TR4)**	*Reference 3* **VCG0124 (*Foc*1)**
Genome size (bp)	53,686,071	55,255,634	61,386,934	61,380,681	61,471,473
Number of Contigs	1239	2824	1362	1362	1371
GC content (%)	50.7	50.5	48.4	48.4	48.5
BUSCO completeness (%)	97.3	96.0	96.4	96.9	98.4
Fragmented BUSCO (%)	0.8	1.2	1.4	0.8	1.4
Missing BUSCO (%)	1.9	2.8	3.0	2.7	3.0
Total genes (Augustus)	19,032	19,310	20,925	17,118	21,842
Average coverage	8x	10x	6x	100x	100x
Accession number	-	-	GCA_000149955	GCA_014282265.3	GCA_011316005.3
Origin of the isolate	Malaysia	Malaysia	Spain	India	India

4.8.3 Molecular identification and characterization of *Foc*1_C2HIR and *Foc*4_C1HIR isolates

The C2HIR and C1HIR were molecularly identified using sequences from the nuclear ribosomal Internal Transcribed Spacer (ITS) region determined from the whole genome sequences. This region is the most commonly chosen genetic marker for the molecular identification of fungal species (Nilsson et al., 2015). Based on the ITS region, the C2HIR and C1HIR were identified as *Fusarium oxysporum* f.sp. *cubense* and both of the isolates shared 99% identity in the gene region with the top matching reference fungal isolates. Phylogenetic analyses grouped the C2HIR and C1HIR isolates within the *Fusarium oxysporum* complex and members of this cluster shared a high degree of sequence identity (90-100%) in the ITS region. Figure 4.41 shows the phylogenetic tree inferred from Maximum Likelihood method on the ITS regions of 33 representative species of *Fusarium* including the isolates of this study and *Ilyonectria radicicola* as the outgroup.

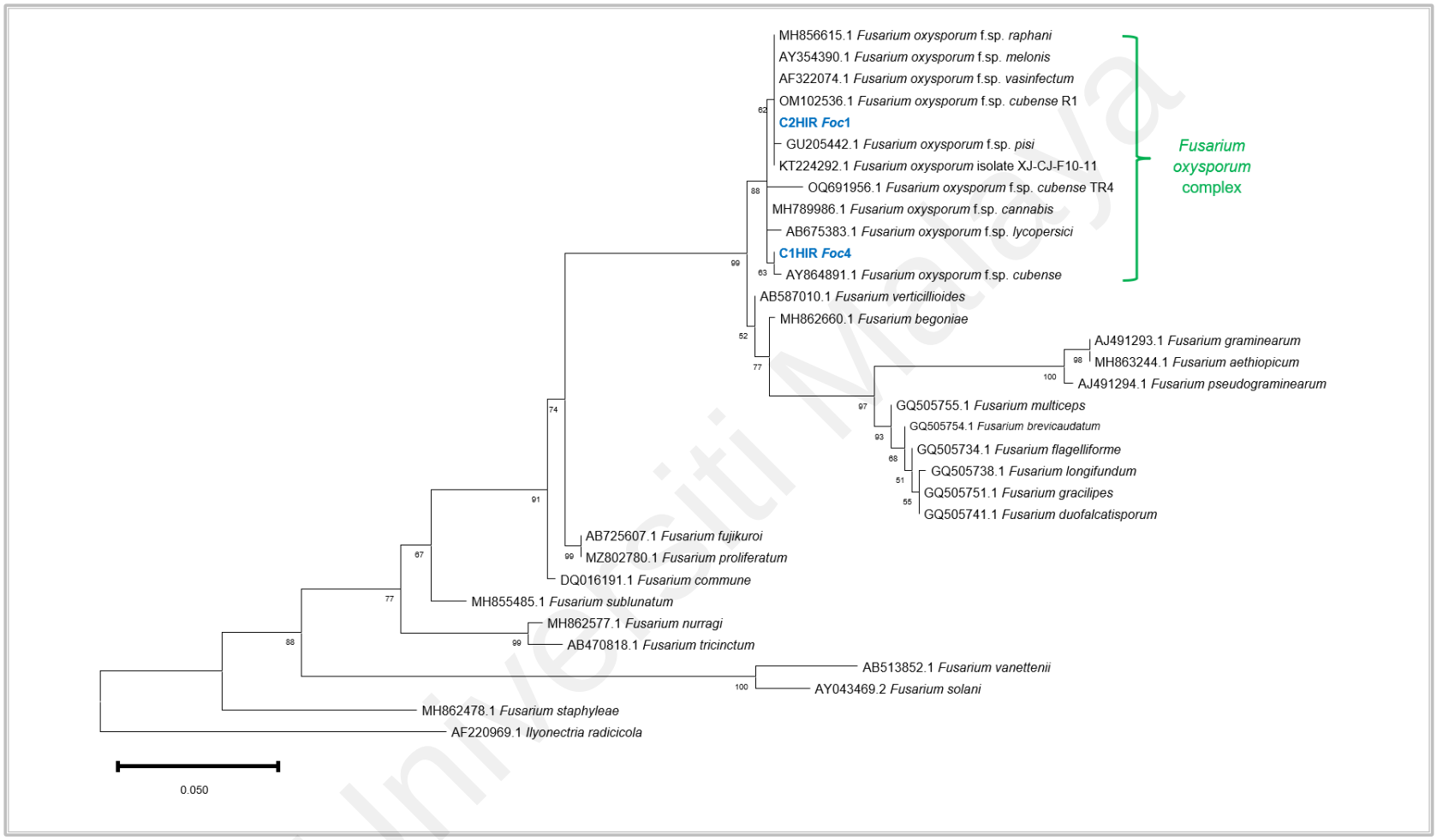


Figure 4.41: Phylogenetic tree inferred from maximum likelihood method on ITS regions of 33 representative species of *Fusarium* including the two isolates of this study and *Ilyonectria radicicola* as the outgroup.

4.8.4 Molecular characterization of *SIX* genes in *Foc*1_C2HIR and *Foc*4_C1HIR genomes

4.8.4.1 *SIX* genes identification via the whole genome annotation

The presence of *Secreted in Xylem* (*SIX*) genes was further validated by annotation of the assembled genomes. The whole genome analysis permitted identification of the *SIX* genes in C2HIR and C1HIR isolates as well as detection of any possible novel *SIX* genes in their genomes. All the *SIX* genes identified in the genomes of isolates C2HIR and C1HIR were tabulated (Table 4.6). A total of six *SIX* genes (*SIX1, SIX2, SIX4, SIX6, SIX8a, SIX9a*) were identified in C1HIR whereas only one *SIX* gene (*SIX9b*) was identified in C2HIR. This shows a difference in the sets of *SIX* genes present in different isolates being studied.

Table 4.6: Comparison of *SIX* genes present in *Foc*1_C2HIR and *Foc*4_C1HIR via whole genome annotation.

***SIX* gene**	***Foc*1_C2HIR**	***Foc*4_C1HIR**
SIX 1	-	✓
SIX 2	-	✓
SIX 4	-	✓
SIX 6	-	✓
SIX7	-	-
SIX8a	-	✓
SIX9a	-	✓
SIX9b	✓	-
SIX 13	-	-

- Absent
✓ Present

4.8.4.2 Gene structure of *SIX* genes

The gene structure of *SIX* genes identified in the genomes of C2HIR and C1HIR isolates were analyzed. *SIX1, SIX2, SIX9a* and *SIX9b* genes were discovered to have one exon and no introns. Whereas presence of two exons and one intron was observed in *SIX4* and *SIX6* genes. *SIX8a* on the other hand, has multiple exons and introns, with a total of four exons and 3 introns in its gene. Table 4.7 summarizes the features of the *SIX* genes C2HIR and C1HIR isolates.

Table 4.7: Summary of *SIX* genes features in C2HIR and C1HIR isolates.

Genes	Features			
	Total gene length (bp)	CDS length	Number of Introns	Number of Exons
	***Foc1* (C2HIR)**			
SIX9b	357	354	-	1
	***Foc4* (C1HIR)**			
SIX1	834	831	-	1
SIX2	699	696	-	1
SIX4	592	402	1	2
SIX6	730	681	1	2
SIX8a	1276	687	3	4
SIX9a	357	354	-	1

4.8.4.3 Sequence analysis of *SIX* genes

The *SIX* gene sequences of C2HIR and C1HIR isolates were BLASTed against three other *Foc SIX* isolates to check for their sequence identity by pairwise comparison. The results showed that *SIX* genes from C2HIR and C1HIR and the other *Fusarium oxysporum* f.sp. *cubense* (*Foc*) isolates shared high sequence identity, ranging from 89% -100% (Table 4.8). All the *SIX* genes from C2HIR and C1HIR isolates shared 100% sequence identity with at least one of the characterized *SIX* genes that were studied for their function in previous studies. This showed that the *SIX* genes identified in the genomes of C2HIR and C1HIR isolates were valid. Remarkably, it was found that the sequence identity between *SIX9a* in C1HIR and *SIX9b* in C2HIR is only 89.1%. This observation underscores the existence of variations among sequences of different races within the same geographical region.

Table 4.8: Pairwise sequence identity (%) of *SIX* genes in C1HIR and C2HIR in comparison with other published *Foc SIX* gene sequences.

Query	*SIX* genes (Query gene length, bp)	Subject isolate	Subject accession length (bp)	Pairwise sequence identity	Accession number
C1HIR	*SIX1* (834bp)	*Foc* Png3-2	789	100 %	MH734307.1
		Foc Jhr2-3	789	99.6 %	MH734309.1
		Foc BRIP62895	834	96.8%	KX434994.1
	SIX6 (684bp)	*Foc* BRIP40340	654	100 %	KX435007.1
		Foc BRIP62895	654	90.8 %	KX438008.1
		Foc R2	153	90.6 %	MW330389.1
	SIX8a (690bp)	*Foc* BRIP40340	521	100 %	KX435011.1
		Foc Png3-2	521	100 %	MH752354.1
		Foc BRIP44012	521	100 %	KX435013.1
	SIX9a (357bp)	*Foc* BRIP62895	357	100 %	KX435015.1
		C2HIR_*SIX9b*	357	89.1 %	-
		Foc BRIP63259	357	89.1 %	KX435016.1
C2HIR	*SIX9b* (357bp)	*Foc* BRIP63259	357	100 %	KX435016.1
		Foc NRRL25609	357	98.6 %	KX435017.1
		Foc BRIP62895	357	89.1 %	KX435015.1

4.8.5 Functional analysis of proteins

4.8.5.1 Clusters of Orthologous Groups of proteins (COG) analysis

The COG analysis grouped the proteins found in the genomes into functional categories. The bar chart below shows the different functional categories where the genes were assigned into and their relative abundances in *Foc*1_C2HIR and *Foc*4_C1HIR isolates in comparison with the reference isolate *Fol*4287. The proteins were categorized into three main COGs, information storage and processing, cellular process and signaling, and metabolism.

A total of 11,740 out of 19,032 proteins in C2HIR and 11,891 out of 19,310 proteins in C1HIR were annotated and grouped into COGs with reference to the COG database. Of the total 11,740 proteins annotated in C2HIR, 1704 (14.5%) were associated with information storage and processing, 2262 (19.3%) were associated with cellular processes and signaling and 4133 (35.2%) were associated with metabolism. In C1HIR, a total of 11,891 proteins were annotated where 1743 (14.7%) were associated with information storage and processing, 2312 (19.4%) were associated with cellular process and signaling and 4115 (34.6%) were associated with metabolism.

The top three most abundant categories in both isolates were G (carbohydrate transport and metabolism) which contains 862 (7.34%) proteins in C2HIR and 847 (7.12%) proteins in C1HIR, Q (secondary metabolites biosynthesis, transport and catabolism) which contains 801 proteins in both C2HIR (6.82%) and C1HIR (6.74%) respectively as well as category O (posttranslational modifications, protein turnover and chaperones) which contains 718 (6.12%) proteins in C2HIR and 737 (6.20%) proteins in C1HIR. Notably, the protein abundance in *Fol*4287 isolate in each of these categories was higher than our isolates (Figure 4.44). In comparison, the remaining functional categories

contain substantially fewer proteins, yet their relative abundances remained consistent between C2HIR and C1HIR.

There was also a set of proteins that have been analyzed as function unknown in both C2HIR and C1HIR, which covers 3641 (31%) proteins in C2HIR and 3721 (31.3%) proteins in C1HIR. Taking the proteins without any functional annotation into account, this outcome underlines that a considerable portion of both C2HIR and C1HIR proteins remain without well-defined functional characterization. Given the reference isolate, *Fol*4287, out of a total of 19,627 proteins annotated, 6231 (31.7%) of them were proteins with unknown function. The percentage of functionally unknown proteins were similar in all the three isolates. Figure 4.42 shows the abundance of proteins in each category for both Malaysian isolates, C2HIR and C1HIR as well as the reference isolate, *Fol*4287.

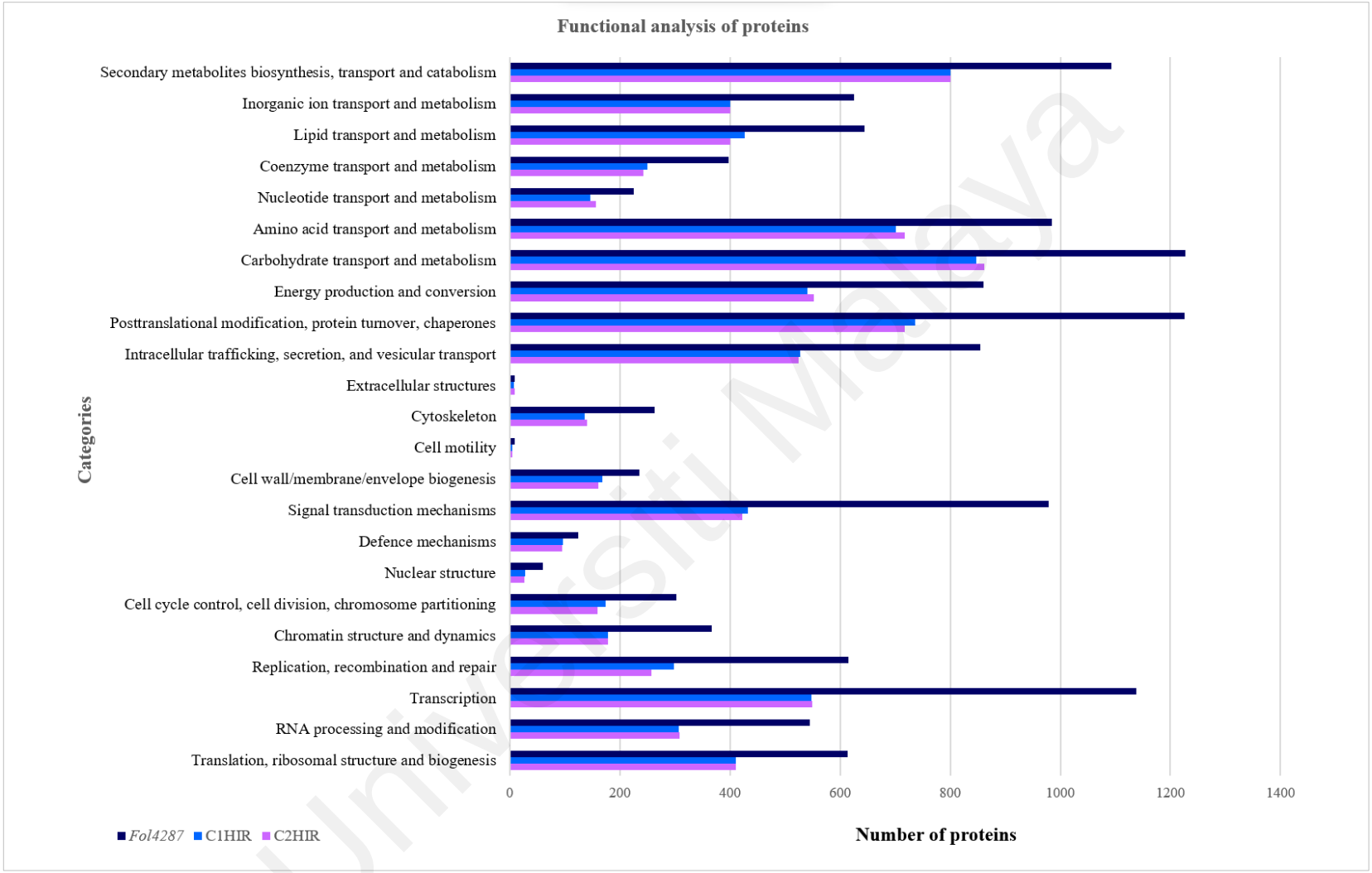


Figure 4.42: Functional comparisons among genome of isolates *Foc*1_C2HIR, *Foc*4_C1HIR and *Fol*4287 based on COG categories. The major categories were listed on the y-axis while the x-axis denotes the number of proteins classified in each category.

4.8.5.2 Pathogen-Host Interaction (PHI) analysis

The protein coding genes annotated from *Foc*1_C2HIR and *Foc*4_C1HIR were functionally annotated with reference to the Pathogen-Host Interaction (PHI)-base to identify the number of pathogenicity- and virulence- related proteins involved in this interaction. PHI-base annotation identified a total of 359 homologs, 178 proteins in C2HIR and 181 proteins in C1HIR with >95% identity making an experimentally verified contribution to the pathogenicity. The results obtained were similar to the reference isolate used, *Fol*4287, that yielded 183 proteins involved in pathogen-host interaction. This set of proteins comprised proteins that act as effector proteins (effector), those that contributed to the pathogen virulence (reduced virulence), those that were essential for pathogenicity (loss of pathogenicity), those that caused lethal effects (lethal), and were involved in negative regulation of virulence (increased virulence). Table 4.9 shows the number of pathogenicity and virulence-related proteins in each fungal isolate.

Table 4.9: Predicted PHI proteins of *Foc*1_C2HIR, *Foc*4_C1HIR and reference (*Fol*4287) isolates.

Mutant Phenotype	Number of PHI-base matches		
	***Foc*1 C2HIR**	***Foc*4 C1HIR**	***Reference Fol*4287**
Loss of pathogenicity	20	20	18
Reduced virulence	139	143	139
Increased virulence	8	8	7
Effector	1	1	8
Lethal	10	9	11
Total	**178**	**181**	**183**

CHAPTER 5: DISCUSSION

5.1 Bioassay experiment

5.1.1 Host plant

'Berangan' cultivars were selected for this study as it is one of the most cultivated, commercially important variety in our county. Comparatively, this variety gives a better yield and has a high demand in the Malaysian market. Studies on *Fusarium oxysporum* f.sp. *cubense* (*Foc*) pathogenesis have mainly focused on Cavendish plants, however, there has been less work reported based on regional cultivars such as 'Berangan'. Hence, studies focusing on 'Berangan' cultivars is vital to advance disease management strategies for a reduced infection rate and better production rate of this commercially important cultivar in our country.

The selection of the developmental and physiological phases of plant material to be utilized is a crucial factor in any plant infection bioassay experiment since disease response and gene expression patterns can differ significantly depending on the maturity level of the cells (Zaidi, 2019). According to Mak and co-workers (2004), plants as young as 2-3 weeks old with a height ranging from 5-7 cm were not suitable to be utilized in bioassay experiments because they were unable to exhibit a steady disease progression. This is probably due to poor vascular tissue development. On the other hand, Karmawan et al. (2019) mentioned that older plants tend to have more mature and lignified xylem vessels, which act as a non-specific barrier against the pathogen. This will eventually affect the accuracy of subsequent expression studies to observe the severe effects of the virulence genes on the banana plantlet, especially for the study of early disease detection. Normally, younger xylems typically have less lignification and are easier for *Foc* to penetrate. Hence, 3-month-old healthy plantlets with at least 3 to 5 leaves, a minimum height of 20cm and stem diameters between 1.0 to 1.5cm were chosen for this study.

Furthermore, 3-month-old plants are easier to handle and can reduce roots damage during the treatment.

5.1.2 Inoculum concentration

The spore suspension culture used for the infection study was standardized to a final concentration of $1x10^6$ spores/ml. The inoculum standardization technique is significant because the optimum desired concentration and reproducibility of the spore suspension influence the bioassay result. Based on a few reported studies, spore concentrations ranging from 10^4 to 10^6 spore/ml have been used (Paul et al., 2011; Guo et al., 2014; Perez Vicente et al., 2014). Lower inoculum concentrations have previously been demonstrated to be inconsistent and might not produce reproducible phenotypic symptoms as indicated by the DSI scores. Conidial concentrations of 10^6 spores/ml are recommended for all bioassays as it has been found to be the most effective at evoking a consistent progression typical for Fusarium wilt disease (Mohd-Yusuf et al., 2019).

Furthermore, it is important to ensure a consistent surface area to volume ratio for inoculum contact. Maintaining consistent spore concentrations in any challenge program is crucial since the variations may have an impact on both the responses and gene expression profiles in subsequent studies (Perez Vicente et al., 2014). In addition, *Foc*'s growth can gradually decrease under unsuitable growth substrates and conditions.

5.1.3 Inoculation technique by double-tray method

The 'Berangan' plantlets of 3-month-old were treated with three types of inoculums (sterile distilled water, *Foc*1 suspension culture, *Foc*4 suspension culture) via the root dipping method. Generally, a reliable infection method should be capable of replicating the natural way in which plants encounter pathogens. In the case of *Foc*, this usually happens through root contact. Thus, the root dipping method is recommended because it is reliable and simple (Mohd-Yusuf et al., 2019). According to Masheva and Todorava (2013), root dipping approach used on pepper and strawberry plants facilitated direct contact between the inoculum and the roots. When employing the root dipping approach, they discovered that the index of infestation was larger than what was observed when non-infected plant materials were planted in contaminated soil.

Besides, the inoculation duration used in this study was 2 hours. This is an effective inoculation time for the fungus to trigger the virulence reaction on banana plantlets. Mohd-Yusuf et al. (2019)'s study on susceptible 'Berangan' cultivars revealed that expected disease progression was produced consistently with an exposure time of two hours. Hence, choosing an appropriate inoculation strategy that minimizes other unanticipated attributes is crucial to avoid obtaining any false positive results.

After the inoculation, the double-tray method was utilized to ensure a safe *Foc* challenge procedure. It is a double compartment arrangement consisting of a small tray that fits snugly into another larger outer tray to which the plants were transferred and maintained at an ambient temperature (28-30 ºC) in the greenhouse. According to Mak et al. (2004), this method eradicates the risk of cross-contamination and enables simultaneous testing of the virulence of multiple *Foc* isolates against various test cultivars. For instance, the drained irrigation water was collected in the outer tray, avoiding contamination at the greenhouse. The drained water was then treated with sodium hypochlorite (NaClO) and discharged.

5.1.4 Sample collection

Sample collection was conducted at four different time points of post-inoculation which are 0 day, 2 weeks, 4 weeks and 5 weeks. This is an established time point used in our laboratory based on a previous study by Mohd-Yusuf et al. (2019). This specific time frame was followed to ensure the consistency and reliability of the results obtained. Additionally, previous studies have reported that, under controlled conditions, *Foc*1 infection displayed external symptoms between the third and fourth week (Sivamani and Gnanamanickam, 1988). While Paul et al. (2011) reported that *Foc*1 can infect Lady Finger (a type of banana variety) as early as three to five weeks after inoculation. These findings support the time frame set for sample collection in this study.

5.2 Pathogenicity test for *Foc*-infected cv. Berangan plants

This assessment was conducted on the 5th week post-inoculation (wpi) plants to determine the level of disease severity caused by *Foc* isolates of Malaysian origin (C2HIR and C1HIR). Only 5th wpi plants were chosen for this assessment because it is the maximum time point used in our study which will disclose the maximum effects caused by the fungal infection. The results obtained revealed that both *Foc*1_C2HIR and *Foc*4_ C1HIR isolates are pathogenic. However, both isolates infected cv. 'Berangan' with different level of virulency. The LSI and RDI of *Foc*4-infected plants were higher compared to *Foc*1-infected plants. The LSI and RDI of *Foc*4-infected plants were 4.25 and 7.25, respectively, whereas *Foc*1-infected plants have scores of 3.25 and 5.0, respectively. This suggested that *Foc*4 is more virulent than *Foc*1 in cv. Berangan (Table 5.1).

Table 5.1: A summary of the DSI and the final translation of cv. 'Berangan' plants after 5 weeks post-inoculation.

Group	Index	Disease Severity Index (DSI)	Final translation
Treatment 2 *Foc*1_C2HIR	Leaf Symptom Index (LSI)	3.5	Highly susceptible
	Rhizome Discoloration Index (RDI)	5.0	
Treatment 3 *Foc*4_C1HIR	Leaf Symptom Index (LSI)	4.25	Highly susceptible
	Rhizome Discoloration Index (RDI)	7.25	

According to Maldonado et al. (2018), three copies of *SIX1* gene are present in the genome of *Foc*4 unlike *Foc*1, which has only one copy. Hence, the higher DSI of *Foc*4-inoculated plants could be due to the gain in copy number in *SIX1* gene that increases the virulency of the fungus. However, in our case, the higher DSI of *Foc*4-inoculated plants could be due to the presence of *SIX1* gene that increases the virulence level which lacks in the *Foc*1 isolate. Furthermore, the difference in the virulence level between both races could also be due to acquisition of additional virulence genes in *Foc*4 isolate, because whole genome analysis from a few previous studies have revealed that the genome of *Foc*4 is slightly larger than *Foc*1 (Thangavelu et al., 2021; Guo et al., 2014). This aligned with the observation in this study where the Malaysian *Foc*4_C1HIR isolate had a slightly larger genome size than the *Foc*1_C2HIR isolate (Table 4.5). Interestingly, according to present study the cv. 'Berangan' plants are highly susceptible to both *Foc* races.

5.3 Identification and validation of *SIX* genes in *Foc* DNA

Before performing PCR amplification for detection of *SIX* genes, the fungal source was amplified with race-specific primers and confirmed as *Foc*1 and *Foc*4 isolates, respectively. The comparison of BLAST results to the acquired *SIX* gene consensus sequences showed that all the sequences had a percentage identity of 95% - 100%, respectively, for each gene. The length of the sequenced *SIX* genes (bp) were also similar to the amplicon size observed in the agarose gel electrophoresis. Hence, the presence of *SIX* genes in the *Foc*1_C2HIR and *Foc*4_C1HIR genomes have been verified and supported by these findings.

PCR amplification *Foc*4 DNA sample with *SIX* gene primers resulted in a single bright band with amplicon size of *SIX1* (923bp), *SIX2* (806-810bp), *SIX4* (930-937bp), *SIX6* (754bp), *SIX8a* (624bp), *SIX9a* (424bp), and *SIX13* (1076-1082bp). It is interesting to note that there was no amplification detected for *SIX7.* The primers used to amplify this gene were sourced from Czislowski et al. (2018), who successfully amplified *SIX7* gene from selected *Foc* isolates. Consequently, the absence of targeted bands in our investigation holds validity. This outcome likely stems from the gene's absence within the genome of the Malaysian *Foc*4_C1HIR isolate. This finding is consistent with the study conducted by Ahmad et al. (2020), stating that *SIX7* was not identified in their Malaysian TR4 isolates, indicating that *SIX7* is not necessarily required for pathogenicity.

On the other hand, *SIX* genes amplified with *Foc*1 DNA showed positive results for *SIX9b* only. Based on a study by Maldonado et al. (2018), *SIX1*, *SIX6*, *SIX9* and *SIX13* were identified in *Foc*1. Interestingly, our Malaysian *Foc*1_C2HIR isolate, only contains *SIX9b*. The absence of other *SIX* genes in this isolate was further confirmed using other published primers by Czislowski et al. (2021). The gene-specific primers did not amplify the respective *SIX* genes; hence, no bands were observed during gel electrophoresis. Similarly, published primers for *SIX6* from Carvalhais et al. (2019) confirmed the absence

of the gene. Additionally, PCR amplification using a *SIX1* gene specific primers that was designed based on gene sequence obtained from NCBI (Accession no: MH734333.1) yielded an unfavourable outcome. Since, the *SIX* genes identified in previous studies were not similar in our situation, this implies that different isolates have their own unique sets of *SIX* genes. van Dam et al. (2016) clarified that every forma specialis possesses a distinct set of *SIX* genes, this indicative that selective pressure from the host could encourage the acquisition of specific groups of secreted proteins, leading to specialization in infecting a limited range of host.

In another perspective, the presence of only one *SIX* gene in this *Foc*1 isolate can be related to the pathogenicity test results. During disease scoring, *Foc*1-inoculated banana plantlets were observed to be less severe than *Foc*4-inoculated banana plants. This could be due to the number of virulence gene present in the *Foc*1_C2HIR and *Foc*4_C1HIR isolates, in this case, *Secreted in Xylem* genes, that increases the virulence level of the pathogen. Furthermore, *Foc*1_C2HIR isolate lacks *SIX1* gene, which have previously been identified as contributing to virulence towards Cavendish banana. Other researches on *Fusarium* isolates that infect tomatoes, cabbage and onions (Widinugraheni et al. 2018), imply that *SIX1* has a broad virulence function in *F.oxysporum*, although not all formae speciales possess a *SIX1* homolog. This highlights the significance of *SIX1* as a virulence factor since its absence in the Malaysian isolate may indicate a less virulent *Foc*1 compared to *Foc*4 isolate. According to Ahmad et al. (2020), high and low disease severity values in leaf wilting and corm discoloration can aid in determining their roles in pathogenicity through gene expression analyses. Therefore, more research focusing on the expression of these *SIX* genes are required to comprehend their roles in pathogenicity.

5.4 Real-time PCR (qPCR)

5.4.1 Validation of reference genes

The precision of qPCR analysis is dependent on stably expressed reference genes for data normalization (Gomes et al., 2018). An imprecise selection of these genes will lead to misinterpreted results. An endogenous gene also known as "housekeeping gene" must be consistently expressed under all experimental and developmental conditions. No single gene has been found to satisfy this requirement, therefore, choosing at least two endogenous gene as reference is recommended to assure more accurate and reliable normalization of gene expression analysis (Gomes et al., 2018; Kumar et al., 2018). In this study, the validation of candidate endogenous genes was conducted on *TEF1α, TUB* and *GAPDH* to confirm their specificity, efficiency and stability. The best reference genes were selected based on two important criteria. Firstly, the gene should be expressed constitutively throughout the infection time points. Secondly, the gene expression must have low standard deviation values across all the studied time points and treatments so that it can be classified as a stable endogenous gene.

During the analysis, a single peak melting curve was obtained for *TEF1α* and *TUB* which indicated that the double stranded DNA products amplified are a single discrete species. On the other hand, the melt curve of *GAPDH* showed multiple peaks indicating multiple products being produced which could be a primer dimer or another gene product. Additionally, the standard deviation of the cycle threshold values for *TEF1α* and *TUB* obtained through Delta CT method during stability test showed a lower standard deviation (SD) value (≤ 1.0) indicating a higher stability of the reference gene. According to Zaidi, (2019), genes that do not exceed the standard deviation threshold value (within 1.5) across all study samples, can be regarded suitable for normalising gene expression.

After interpreting these results, *TEF1α* and *TUB* was selected as the suitable endogenous gene for this study. It is crucial to understand that interpreting the qPCR data obtained is challenging because there is a higher potential of error given the numerous stages of sample preparation and processing. These includes variation in initial sample amounts RNA extraction, reverse transcription to cDNA, cDNA storage and quality, primer selection, experimental design, and statistical analysis. Therefore, to account for these variations, qPCR data must be normalised against an endogenous gene (Kumar et al., 2018).

5.4.2 Gene expression studies on *SIX* genes

In general, management strategies are used to control diseases, and for the strategies to be sustainable and effective, they must be based on actual understanding and knowledge of the underlying biology of each specific plant-pathogen interaction. This entails understanding the pathogen's strategies for attacking the host plant, how well the plant can defend itself from such attacks, and how the environment affects the interactions (Daayf et al., 2012). In this regard, the focus has to be on effector proteins that are critical for virulence of a pathogen and in the case of *Foc*, it is important to look further into *SIX* genes as they can be a potential biomarker for early detection of the Fusarium wilt disease. Jenkins et al. (2021) stated that determination of expression of the *SIX* genes is important as they serve indication if they play a function in infection facilitation.

Gene expression studies were conducted on *Foc*1- and *Foc*4-infected banana root samples over four time points which are 0 day, 2 weeks, 4 weeks, and 5 weeks. These time points were studied in order to monitor early and late events during infection and to focus on the disease progress. This period encompasses the transition from biotrophy to necrotrophy and involves events at various phases of pathogen colonization and host

defence. *Fusarium oxysporum* is a hemi-biotrophic pathogen, where it shifts from biotrophic to necrotrophic lifestyle as the infection progresses (Pradhan et al., 2021). The increased pressure from the host defense and enhanced requirement for nutrients to support the pathogen's aggressive growth are anticipated to be the factors that induce the transition from biotrophy to necrotrophy (Kabbage et al., 2015). This study designates the time points of 0 day and 2 weeks as the biotrophic phase, the initial stage of infection. During this phase, pathogens employ stealthy methods to evade plant defenses. Subsequently, the necrotrophic phase ensues at the 4th and 5th week, marked by rapid cell death in hosts and triggering significant molecular responses from the plant.

In the present study, a total of five virulence-associated target genes (*SIX1, SIX6, SIX8a, SIX9a, SIX9b*) were shortlisted to look into their expression pattern during *Foc* inoculation on cv. 'Berangan' plantlets at specific time points. These genes were initially chosen for gene expression studies for their necessity towards virulence as well as those previously reported to be identified in both *Foc*1 and *Foc*4. The significance of these *SIX* genes has been also evaluated in our study based on the gene expression analysis, and it has provided some insights on the virulence genes in *Foc*1 and *Foc*4 that were mediated during the plant pathogen interaction. In *Foc*4, a similar expression pattern was observed for all the genes being studied. Generally, all *SIX* genes (*SIX1, SIX6, SIX8a, SIX9a*) showed a significant upregulation on the 2nd and 5th week but only a slight upregulation on the 4th week.

Previously, most of the host-pathogen interactions were explained in the context of pathogens attacking their hosts, with the hosts subsequently responding to the attack. Scientists applied this model to test various hypotheses and discovered the majority of what we know today about pathogens' invasion strategies and the mechanisms in plants that lead the plants to either resist or succumb to the invasion (Daayf et al., 2012). The gene expression pattern observed in this study can be explained by the pathogen counter

defense mechanism exerted by *Foc.* Counteracting plant defense responses plays a vital role in plant-microbe interactions, particularly in cases involving biotrophic and hemi-biotrophic pathogens which depend on living plant tissues to establish a successful infection.

Upon the initial infection by *Foc*, the host plant's defence genes exhibit a delayed response, thereby permitting the heightened expression of fungal virulence genes. Later, when the plant defense mechanism is activated, the fungal virulence genes expression decreases, implying the plants attempt to eradicate the pathogen. However, *Foc* was able to counteract causing a significant upregulation on the 5th week. The upregulation of the *SIX* genes on the 5th week is probably due to the pathogen's counteract mechanism that could suppress the plant's defence mechanism. This is similar to the case of *Phytophthora infestans* in potato, where highly aggressive *P. infestans* suppressed the expression of potato defence genes, *pal1* and *hmgr2*, resulting in a decrease in phytoalexins. This was seen as a strategy employed by *P. infestans* to counteract the defense mechanisms of potato (Daayf et al., 2012; Wang et al., 2008).

In contrast, the expression of *SIX9b* gene in *Foc*1 was downregulated on the 2nd and 4th week but significantly upregulated on the 5th week post inoculation. *SIX9b* could possibly be a late response virulence gene that is expressed at the later stage of infection. Kahmaan et al. (2001) pointed out that the upregulation of late-stage genes by necrotrophic pathogens as well as facultative biotrophs may be attributed to the action of hydrolytic enzymes that liberate the release of large quantity of nutrients from plant tissue, facilitating extensive pathogen proliferation and production of mature spores.

Apart from that, similar case was also seen in human where the *SAP2* virulence gene from *Candida albicans* was not expressed during early stage of infection but was highly elevated at later stage after dissemination into deep organs. Since the induction was observed after intravenous infection instead of intraperitoneal infection, this indicates that

the environmental changes within the organs that influence gene expression depend on the mode of infection (Staib et al., 1999). Therefore, in the present study, it is possible to hypothesize that the environmental changes within the banana plantlets may have had an impact on the expression pattern of *SIX9b* based on its adaptability to proliferate vigorously in response to a sudden environmental change.

SIX proteins are expected to be emitted into the plant host's xylem to ensure the fungal pathogen's virulence (Rep et al., 2002; Houterman et al., 2007; An et al., 2019). The *SIX* genes are assumed to be active when the fungus comes into contact with the host, and they are frequently associated with disease manifestation. Moreover, plant pathogen effectors are primarily expressed during the in vivo colonization and exhibit different expression profiles at different stages of infection (O'Connell et al., 2012; An et al., 2019). Based on a study by An et al. (2019), the transcription levels of the *Foc*TR4 *SIX* genes were extremely low when cultured in vitro, but all *SIX* genes were significantly induced after plant inoculation. They added that the expressions of *SIX1a, SIX1b, SIX1c*, *SIX8* and *SIX9* increased throughout the infection, implying that these 5 genes play significant roles in the colonization and infection of *Foc* TR4 to banana plants. Furthermore, Widinugraheni et al. (2018) observed that knocking out *SIX1a* in *Foc*TR4 significantly lowered virulence towards Cavendish banana. These findings are in accordance with our gene expression study where *SIX1*, *SIX6, SIX8a* and *SIX9a* showed high expression in *Foc*4 after inoculation.

In another study by Armitage et al. (2018), *SIX9* was identified as one of the 20 highest expressed genes on a pathogenic *Fusarium oxysporum* f.sp. *cepae* isolate during infection on onions. Our result is consistent with the published data because *SIX9* showed high expression during *Foc*4 infection in banana plants. In the case of *SIX6,* Gawehns et al. (2014) demonstrated that *FolSIX6* transcripts were detectable in planta as early as 96hpi, but the in vitro expression was not examined. It has been said that *Fol* effectors, such as

SIX6, interfered with cell death and altered the plant's immunological responses. Our findings on the upregulation of *SIX6* in *Foc*4-infected banana plants is supported by Poon et al. (2019), stating that *FocSIX6* has a potential function both in planta and during *Foc* metabolism, as evidenced by its constitutive expression in vitro and significant upregulation in planta.

Taken together, this research unveiled distinct expression patterns in *Foc*1-infected and *Foc*4-infected cv. 'Berangan' plants, indicating varying levels of pathogenicity among the isolates as well as the potential virulence genes suitable for early detection of Fusarium wilt disease. Notably, the expression dynamics of *SIX1* and *SIX9a* during the 2nd week in *Foc*4-inoculated plants position them as promising candidates for biomarker development in early disease diagnosis. Conversely, *SIX8a* emerges as a valuable component for race-specific detection as it is exclusively present in *Foc*4 and absent in *Foc*1. Indeed, the meticulous choice of specific time points for early detection strategies is paramount. The unique expression pattern of genes like *SIX9b* in *Foc*1, observed at a later stage, underscores the necessity for tailored approaches accommodating diverse characteristics of pathogen races. Early identification becomes imperative, as observable symptoms manifest only after the infection spreads to other parts of the plant. Therefore, this study holds transformative potential for the agricultural industry, offering insights for the development of future on-site detection methods, thus serving as a game changer in disease management.

5.4.3 *SIX* genes as potential gene expression-based diagnostic biomarker

Increased comprehension of gene biology leads to the development of promising marker candidates for more precise diagnosis (Novelli et al., 2008). In diagnostic research, an effective and validated gene expression-based biomarker discovery procedure can be a very useful necessary tool (Monforte and McPhail, 2005). A biomarker is a DNA sequence that causes disease or is linked to a disease's susceptibility. They can be used to create genetic map of the targeted organism. Diagnostic biomarkers can detect and confirm the presence of disease or a condition of interest (Vellan et al., 2022). Metabolomic biomarkers have demonstrated their ability to predict phenotypic features prior to their occurrence. Metabolomics is a powerful tool in this domain since it can characterise and measure many metabolites simultaneously. Comparatively, gene expression based-biomarker studies involve a targeted gene expression analysis, followed by the construction of a reference gene for that specific sample set and one candidate gene will be identified as a potential susceptibility-associated biomarker (Maia et al., 2020).

Based on previous studies, plant pathogen effectors are primarily expressed during in vivo colonization, with variable expression profiles at different phases of infection (O'Connell et al., 2012; An et al., 2019). Genes with significantly higher or lower expression in cells under normal versus infected conditions are typically identified using a variety of techniques for gene expression-based biomarkers development (Harris et al., 2015). Gene expression-based biomarkers are commonly used in cancer diagnostics. For instance, Bhalla et al. (2017), examined for expression-based gene biomarkers that could distinguish between individuals with clear cell renal cell carcinoma (ccRCC) in the early and late stages. They analysed a total of 523 samples to find the differentially expressed genes in the early and late stage of ccRCC. Additionally, they have also attempted to develop gender-specific models that could improve prediction performance for a given gender.

This approach can be applied to our study after further verification is conducted to prove this candidate *SIX* gene biomarkers as actual diagnostic biomarkers. Early diagnosis is a crucial strategy to minimize disease induced crop damage in crops during growth and to maximize production to ensure agricultural sustainability (Fang and Ramasamy, 2015). *SIX* genes in *Foc*1 and *Foc*4 can be used for different stages of detection since they exhibit different patterns of expression. Therefore, in our case, *SIX* genes in *Foc*1 and *Foc*4 satisfy the requirement for early disease detection at different time points since they are significantly expressed in different infection stages.

It is worth noting that our study highlights a crucial observation where cv. Berangan plants exhibit high susceptibility to both *Foc*1 and *Foc*4 isolates. This underscores the paramount importance of prioritizing disease detection strategies. Based on a previous study, *SIX* genes were substantially upregulated upon plant inoculation compared to invitro (An et al., 2019). This supports the decision to identify candidate *SIX* gene biomarkers based on the gene expression analysis. According to Fraser-Smith et al. (2014) *SIX8* can distinguish race 4 isolates from race 1 and 2 isolates, and variations in SNP of *SIX8* could additionally differentiate isolates of tropical and subtropical race 4. Thus, race specific control measure can be established to facilitate the development of a strategic molecular framework that entails sampling of the plants until the disease is diagnosed via identified biomarkers. Hence, we can hypothesize that *SIX1, SIX8a* and *SIX9a* can be utilized as a potential gene expression-based biomarkers to detect early infection in banana since their expression patterns change significantly after *Foc*4 infection. Similarly, *SIX9b* can be considered as a candidate biomarker in the case of *Foc*1 infection.

5.4.4 Early disease detection strategies using *SIX* genes

This study highlights the critical importance of implementing effective disease management and prevention strategies to mitigate the impact of Fusarium wilt of banana. Drawing on our research findings, a series of key measures can be adopted to screen, identify, and manage the disease proactively. Initiating pre-planting soil testing emerges as a pivotal step, involving the examination of soil samples for the presence of *Foc*1 or *Foc*4 using race-specific primers before introducing banana plants to a new farm or field. This approach facilitates early detection of the pathogen in the soil, enabling timely intervention.

Upon confirming the presence of *Foc* in the soil, the utilization of identified potential biomarkers, *Secreted in Xylem* (*SIX*) genes, becomes instrumental for additional screening of pathogenic isolates at specified time points. Given *Foc*'s reliance on a host for proliferation and its ability to remain dormant in the absence of a host plant, implementing crop rotation proves to be a beneficial strategy for minimizing the emergence of Fusarium wilt in a specific field.

Following the planting of banana plants, routine checks aligned with the suggested early detection time points are essential. If disease symptoms are identified, molecular detection methods can be employed to confirm the presence of *SIX* genes, allowing for the quarantine of affected plants and the surrounding area to prevent further disease spread. Hence, by diligently applying these measures, banana growers can proactively manage Fusarium wilt, curbing disease spread and safeguarding their crops, thereby contributing to the sustainable cultivation of bananas.

5.5 Whole genome sequencing and analysis

5.5.1 Genome sequencing and general features

The genomes of *Foc*1_C2HIR and *Foc*4_C1HIR isolates that were pathogenic towards the banana cultivar 'Berangan' in Malaysia were sequenced and characterized in this study. In the process, *de novo* genome sequence assembly was performed as the approach is effective in generating new sequence assemblies for previously uncharacterized genomes and to identify the genome sequence of individuals in a reference-unbiased way (Simpson and Durbin, 2011). The C2HIR and C1HIR genomes were sequenced with Illumina Novaseq sequencer (150-bp paired end read setting) and de-novo assembled. The results revealed that the average quality scores across all bases for both C2HIR and C1HIR genomes were in the range of 34 to 36. The Q-score for this range is Q30 which corresponds to a Phred score of 30. This result indicates that 99.9% of the nucleotides in the genome were correctly sequenced, with only one error occurring per 1000 bases (https://www.illumina.com/). The occurrence of larger standard deviation in quality scores towards the end of the sequencing bases at positions 130 to 150 for the reverse reads was observed. This is because, the quality of the bases generated by sequencers tends to drop down towards the 3' end of a sequencing run due to limitation of the technology, in terms of stability of reagents (Bao et al., 2014).

Although the assembled genome size of C2HIR and C1HIR were comparable but they were smaller than the reference genomes. Interestingly, there were noticeable differences in the *Foc* genome of Malaysian isolates, specifically slightly larger genome size, when compared to well-known *Foc* genome assemblies reported from other parts of the world. For instance, based on a study by Yun et al. (2019), *Foc*1 genome of China origin had a genome size of 48.56 Mb and *Foc*4 genome had 48.16 Mb. In another study by Asai et al. (2019), *Foc*4 genome of Japan origin was 51.4 Mb. The differences in the genome sizes of the fungus at different parts of the world could be due to variations in the

amplification, deletion, and divergence of different repetitive sequences, including the transposable elements, which constitute a large fraction of the genome (Boulesteix et al., 2006). On the other hand, Raman et al. (2021) stated that the difference in genome size between Indian *Foc* isolates (in the range of 61.4 Mb-63.2 Mb) and other *Foc* isolates is likely attributable to the fact that libraries were built with larger inserts and more mate-pair information were obtained from them, allowing the connection of contigs into scaffolds more easily.

5.5.2 Molecular characterization of *SIX* genes in *Foc*1_C2HIR and *Foc*4_C1HIR

Genome annotation revealed the presence of different *SIX* genes in the Malaysian C2HIR and C1HIR isolates of this study. The annotation results corresponded to the results obtained via PCR amplification of *SIX* genes using gene-specific primers earlier in this work. However, *SIX13* was not identified in the annotated genome of C1HIR isolate despite its detection during PCR amplification. This might be due to the reason that the gene had lost in between contigs as the genomes obtained for C1HIR was only in draft status. Additionally, stringent quality filtering was performed prior the genome assembly. It was possible that the *SIX13* gene was located on low coverage raw sequence reads that were filtered out before genome assembly. Mende at al. (2012) carried out a study by assembling the datasets with and without quality filtering. It was found that quality filtering excluded a large amount of the data, but greatly increased the contig lengths and improved the accuracy of resultant assemblies. This shows the importance of effective quality control as the Illumina assemblies significantly improved after stringent trimming and quality filtering.

During the genome annotation, only *SIX9* gene was commonly identified in both *Foc* races, *SIX9a* in *Foc*4_C1HIR and *SIX9b* in *Foc*1_C2HIR. *SIX9a* and *SIX9b*, being homologs, has a gene length of 357 bp exhibiting a sequence similarity of 89% (Table 4.8), with 318 out of 357 bases displaying similarity. This highlights the presence of genetic variations or differences within these homologous genes across different races of the same forma specialis. The divergence in nucleotide arrangements between homologous genes in different races underscores the adaptability of pathogens like *Foc.* These variations, likely influenced by evolutionary pressures, may significantly contribute to differences in virulence, host specificity and other aspects of pathogenicity.

Analysis on the *SIX* gene structures revealed number of introns and exons present within the genes. In the present study on *Foc*4_C1HIR, *SIX6* and *SIX8a* had one intron and three introns, respectively, while *SIX1* and both *SIX9a* and *SIX9b* lacked introns. Based on the finding by Poon et al. (2019), two introns were present in *FocSIX8a*, while most of the *SIX* genes identified were intronless suggesting that the multi-exons feature of *FocSIX8a* shows a possibility of alternative splicing and isoforms. Alternative splicing (AS) is a cellular process that is well known in higher eukaryotes and in fungi, the involvement of AS in gene expression and its effects on multi-cellularity and virulence is of great interest (Grutzman et al., 2013). The previous studies revealed that AS could regulate virulence. For instance, in a study by Jeon et al. (2022) to assess the potential AS involvement during infection and discover genes related to pathogenesis that were regulated by AS, revealed that AS could influence the translation of genes involved in virulence (plant cell wall degrading enzymes) and host adaptation (autophagy).

Another study by Rodriguez-Kessler et al. (2012) demonstrated that *UmRRM75*, a multiexonic gene with four introns and five exons gene, which encodes a protein with three RNA recognition motifs, affects the morphogenesis and virulence of the plant fungal pathogen *U. maydis*. This is similar to the case in our study, where *SIX8a* has a

multiexonic feature suggesting the involvement of AS in the virulence of this particular gene. Moreover, the inclusion of the *SIX8* gene in *Foc*4_C1HIR could be an additional contribution to this isolate's increased level of virulence compared to *Foc*1_C1HIR, which lacks the *SIX8* in their genome. This finding corresponds to our pathogenicity test, which exhibited higher DSI score of *Foc*4-inoculated 'Berangan' plantlets compared to *Foc*1-inoculated plantlets. However, AS remains insufficiently characterized in plant-pathogenic fungi and warrants more comprehensive investigation, despite some prior studies that have generally explored the role of AS in regulating virulence. Regrettably, systemic studies addressing the pivotal question of the role of AS in the lifestyle of plant pathogenic fungi are notably absent. Hence, further research should be undertaken to unveil the specific contribution of alternative splicing to the diverse lifestyles exhibited by fungal pathogens (Ibrahim et al., 2020).

5.5.3 Functional analysis of proteins

5.5.3.1 Cluster of Orthologous Groups of proteins (COG) analysis

The accomplishment of a complete genome study critically depends on reliable genome annotation which involves precise gene identification, accurate determination of gene boundaries and functional annotation of gene products (Galperin et al., 2019). The fraction of proteins from a specific genome allocated to particular COG functional categories has proven to be a valuable feature of whole genomes and has been endorsed by the Genome Standards Consortium as a crucial attribute for newly sequenced genomes (Galperin et al., 2019).

The COG analysis has assigned the proteins in the genomes of both *Foc*1_C2HIR and *Foc*4_C1HIR isolates into a few categories. The group with highest number of proteins in both isolates was in G (carbohydrate transport and metabolism). These proteins are important for basic cellular function and central metabolism, i.e., energy production because they participate in glycolysis to produce ATP (Sun et al, 2014). The second highest, category Q involving secondary metabolites, transport and catabolism. Fungi employ secondary metabolites for various purposes, including defence against predators and competitors, chemical communication, and, in the case of pathogenic fungi, to manipulate their animal and plant hosts (Brakhage, 2013; Haas, 2015). This category has 801 proteins in both C2HIR and C1HIR suggesting the importance of focusing on the proteins in this category to identify any potential metabolites that can be used as a marker for detection of fungal disease on host plants, in our case, during the infection of *Foc* on 'Berangan' plants.

The third most important category was category O involving proteins for posttranslational modification, protein turnover and chaperones. Generally, posttranslational modification proteins play important role in regulating cellular structure and many other biological processes and the chaperones assists protein folding in the cell under physiological and stress conditions (Ramazi and Zahiri, 2021; Chatterjee et al., 2018). Another category that drew attention was category U, intracellular trafficking, secretion and vesicular transport. This category has proteins involved in secretion, which could be also secreted proteins that could form a secretome. The secreted proteins might play a role in fungal virulence to establish a successful infection on host plant, for instance, in our study, Secreted in Xylem (SIX) is a type of secreted protein, that plays a major role in *Foc*-banana pathogenic interaction.

Besides, there were a large number of proteins grouped as function unknown (category S) in both C2HIR and C1HIR isolates. This indicates there are more numbers of uncharacterized/hypothetical proteins in both isolates. These are potentially the genes that are undergoing rapid evolution and thus displaying high variation that did not match the BLAST result for the given criteria (Raman et al., 2021). Hence, further research is needed to study and analyse these proteins so that we can uncover any novel proteins that could be involved in the virulence function of the fungus that causes severe effects on cv. 'Berangan' plants. According to Galperin et al. (2019), these uncharacterized proteins are particularly useful because they reflect the state of knowledge regarding protein function on the proteome level and enable tracing the advancements in experimental characterization and computational analysis of widespread protein families.

On the other hand, the number of proteins in each category in the reference isolate, *Fol*4287, was higher compared to those in Malaysian C2HIR and C1HIR isolates. This is probably due to the larger genome size of the reference genome, ~61.4Mb, compared to C2HIR and C1HIR isolates with ~53Mb-55Mb genome size. However, the percentage of protein abundance in the three main COG categories are similar in all the three isolates.

5.5.3.2 Pathogen-Host Interaction (PHI) analysis

During plant-fungi interaction, fungi can secrete numerous proteins aimed at manipulating the immunity and physiology of their hosts. This manipulation serves various purposes, such as evading host recognition, suppressing plant defences, facilitating infection, and triggering plant cell death (He et al., 2021). To find potential virulence-associated genes, a whole genome BLAST analysis was performed with reference to the pathogen-host interaction (PHI) gene database. The analysis revealed a total of 178 and 181 putative virulence-associated genes in C2HIR and C1HIR,

respectively. This set of proteins comprised proteins that act as effectors, those that contribute to pathogen virulence, those that are essential for pathogenicity, those causing lethal effects and those involved in negative regulation of virulence.

Effectors are essential fungal pathogenic factors that promote infection or elicit defence responses on host plants. PHI-base classifies a protein as an effector based on its ability to trigger a hypersensitive reaction via a compatible interaction between the protein and the host plant's corresponding receptor protein (Khairi et al, 2022). Most of these proteins were predicted to have pathogenicity-related phenotypic outcomes in mutation experiments (He et al., 2021). In our study, there was only one effector protein identified each in C2HIR and C1HIR whereas eight effector proteins were identified in the reference isolate with >95% identity. Notably, there were additional effector proteins (plant avirulent determinant) that has been validated in the reference isolate such as *SIX5, Avr2, Avr3, SIX2* and *SIX6*. The most dominant mutant phenotype identified was for reduced virulence proteins in both isolates, 139 proteins in C2HIR and 143 proteins in C1HIR. These are the proteins that have been identified to play a role in the pathogen's virulence through either gene deletion, gene disruption, gene complementation or gene silencing experimental techniques.

The number of virulence associated genes (VAGs) in *Foc*1_C2HIR and *Foc*4_C1HIR is lesser to that of other sequenced fungi in previous studies, for instance, *Foc*4 isolate II5 has 357 VAGs (Guo et al., 2014). This could be due to the parameters that has been set especially, in our study, the cut of standard was set to a percentage identity of >95% to predict a more accurate outcome in terms of their similarity. Based on previous study by Guo et al. (2014), the search of the PHI database yielded 15 well-characterized VAGs from *Fo,* including *ARG1, CHS2, CHSV, CHS7, FMK1, FGA1, FGA2, FGB1, FOW1, FOW2, foSNF1, FRP1, GAS1, PacC* and *SIX1.* This indicates that there are conserved VAGs and pathogenesis among the different formae speciales of *Fo*. This finding is in

accordance with our current study, where the PHI-base annotation of VAGs identified the presence of all 15 proteins in C1HIR. In contrast, only 14 proteins were identified in C2HIR. This discrepancy arises from the absence of *SIX1* in the C2HIR isolate whereas all 15 VAGs were identified in the reference isolate. Table 5.2 shows the details of the well-characterized VAGs in *Fo*.

Table 5.2: The details of well-characterized virulence-associated genes of *Fusarium oxysporum* species identified in *Foc*1_C2HIR and *Foc*4_C1HIR isolates.

Pathogen gene	Gene function	Mutant phenotype	Pathogen species	Disease	Host species	Experimental technique
ARG1	Argininosuccinate lyase	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption; gene complementation
CHS2	Chitin Synthase	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption
CHSV	Chitin Synthase	Reduced virulence	*Fusarium oxysporum*	Fusarium wilt	*Solanum lycopersicum*	Altered gene expression / gene regulation: silencing; other evidence: transgenic plants.
CHS7	Chitin Synthase	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption
FMK1	MAP kinase	Loss of pathogenicity	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption; gene complementation
FGA1	G alpha protein subunit	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption
FGA2	G alpha protein subunit	Loss of pathogenicity	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption

Table 5.2, continued.

Pathogen gene	Gene function	Mutant phenotype	Pathogen species	Disease	Host species	Experimental technique
FGB1	G beta protein subunit	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption
FOW1	Mitochondrial carrier protein	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption; gene complementation
FOW2	Zn (II) 2Cys6-type transcriptional regulator	Reduced virulence	*Fusarium oxysporum*	Fusarium wilt	*Solanum lycopersicum*	Gene deletion: full
foSNF1	Protein kinase	Reduced virulence	*Fusarium oxysporum*	Vascular wilt	*Arabidopsis thaliana*	Gene disruption
FRP1	F-box protein	Loss of pathogenicity	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene disruption; gene complementation
GAS1	Glucanosyltransferase	Reduced virulence	*Fusarium oxysporum*	Wilt	*Brassica rapa*	Gene deletion: full
PacC	Zinc finger transcription factor	Increased virulence (hypervirulence)	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene mutation; gene complementation
SIX1	Effector protein	Effector (plant avirulence determinant)	*Fusarium oxysporum*	Vascular wilt	*Solanum lycopersicum*	Gene deletion; gene complementation

5.6 Challenges and future directions

The interaction between plant genotypes and pathogens, as well as environmental factors, appear to have a significant impact on the Fusarium wilt of banana development (Groenewald et al., 2006). As a typical necrotrophic fungus, *Foc* can persist in soil for extensive stretches as thick-walled chlamydospores prior to infecting banana (Di Pietro et al., 2003). Destruction of diseased plants, soil rotation with paddy rice, utilization of plant materials that are contamination-free and fungicide usage are the main control measures for Fusarium wilt disease. Nonetheless, these control techniques still cannot prevent *Foc* infection on banana effectively. In addition, high sterility triploid varieties make it extremely difficult to foster wilt-resistant banana cultivars using conventional breeding methods (Bai et al., 2013).

Besides, due to the variety of *formae speciales* and races in FOSC, pathogenicity assays are tedious and difficult to discriminate between pathogenic and non-pathogenic isolates (Recorbet et al., 2003). Standard molecular loci-based methods for fungal phylogenetics, on the other hand, are compelled by a weak association between pathogenicity and phylogenetic relationships (Fraser-Smith et al., 2014). Methods that make use of specific gene sequences linked to pathogenicity such as *SIX* genes can be utilized to overcome the challenges mentioned above (Recorbet et al., 2003; van der Does and Rep, 2007; Lievens et al., 2008; van Dam et al., 2018). According to Jangir et al. (2021), *SIX* genes have been used as an ideal pathogen identification marker. By identifying unique pathogenicity factors in *Foc* pathogenic isolates, researchers can gain a better comprehension of how this pathogen causes infection in bananas as well as a source for quickly recognizing pathogenic isolates (Ghag et al., 2015). Therefore, improved understanding of the *SIX* gene's role in *Fo* virulence and variability has

important implications for the development of a molecular framework and disease management approaches (Jangir et al., 2021).

Currently, different methodologies have been used to control this damaging pathogen; be that as it may, the utilization of resistant cultivars is the most satisfactory and economical to manage Fusarium wilt of banana (Michielse and Rep, 2009). Even though scientists have been endeavouring to increase banana resistance to Fusarium wilt disease, commercially available varieties are currently restricted (Dong et al., 2019). Recent reports on genomic approaches in understanding the interactions between *Foc* and banana have started to reveal insights into the potential outcomes of investigating new techniques for the further development of resistant varieties (Mohd-Yusuf et al., 2019). Hence, research on the interaction of *SIX* genes in *Foc* and banana will bring new insights into the components by which the host plant reacts to the fungal infection and could prompt the disclosure of proficient approaches to combat banana Fusarium wilt disease.

This current research can be expanded to include proteomics, i.e., the study of protein-protein interactions and regulatory pathways involved during the infection of *Foc* on banana plants. This is critical for comprehending cell physiology in both normal and pathological states. Having said that, the comparison of Malaysian *Foc*1_C2HIR and *Foc*4_C1HIR isolates has provided a better understanding of the putative proteins involved in each COG category, which can be further explored to look into proteins that plays vital roles in pathogen-host interaction. Once this interaction is understood, gene editing technologies can be applied to produce banana plants that are resistant to Fusarium wilt pathogen infection. However, since this technology will consume a longer time to be implemented, hence, this emphasises the significance of early detection that could serve as the quicker option to address the lethal disease.

Subsequently, this study highlights one of the prospective applications for the future, which is the development of an on-site detection method utilising the identified biomarkers. Although pathogen identification is often accomplished by molecular techniques, however, there is a demand for on-site immediate identification. To the best of our knowledge, no detection kit is currently being used in Malaysia's agricultural industry. When it comes to the types of crops grown in Malaysia, the banana cv. 'Berangan' is one of the most cultivated, and the disease has caused a significant reduction in banana production. The identification of candidate biomarkers, coupled with *Foc* genome analysis in this study, along with nanotechnological advancements, paves the way for a promising future in the development of a rapid on-site detection kit for Fusarium wilt of banana. Hence, this technological innovation holds the potential to make a substantial difference in our community, benefiting farmers and contributing to the sustainability of our nation's food security.

CHAPTER 6: CONCLUSION

Secreted in Xylem (SIX) genes became the central focus because of their potential as biomarkers. Therefore, comprehending the molecular basis of virulence in the pathogen as well as its mechanism is crucial for advancing further research to combat this disease effectively. The pathogenicity test has revealed distinct virulence levels among different races of *Foc,* with *Foc*4 demonstrating higher virulence in comparison to *Foc*1. Notably, the cv. 'Berangan' plants exhibited high susceptibility to both Malaysian *Foc*1 and *Foc*4 isolates, underscoring the need for targeted strategies to address the virulence variations within these pathogen races.

Based on the acquired results, *SIX1, SIX2, SIX4, SIX6, SIX8a*, *SIX9a* and *SIX13* were identified to be present in *Foc*4 while *SIX9b* was identified in *Foc*1. The presence of these *SIX* genes in *Foc* has been conclusively validated via sequencing analysis. Notably, the expression profiles of *SIX1, SIX6, SIX8a* and *SIX9a* exhibited upregulation during the 2nd and 5th week in *Foc*4-inoculated plants. This could be due to the pathogen's counteract mechanism to overcome the plant's defense. On the other hand, *SIX9b* in *Foc*1 demonstrated upregulation during the 5th week indicating a delayed expression pattern. Thus, a tailored approach should be devised for early detection at specific timepoints, specifically targeting the 2nd week for *Foc*4 and the 5th week for *Foc*1. In this context, *SIX1* and *SIX9a* emerge as promising candidate biomarkers for early disease detection in *Foc*4-infected plants while *SIX8a* can be harnessed for race-specific detection. Concurrently, *SIX9b* stands out as a valuable biomarker for use in *Foc*1-infected plants. This customized strategy capitalizes the unique expression patterns of these genes, optimizing their utility of precise and timely identification of Fusarium wilt in different pathogen races.

The comprehensive analysis of whole genome sequencing unveiled the commendable quality of the *Foc*1_C2HIR and *Foc*4_C1HIR isolates of Malaysian origin, characterized by genome sizes of 53 Mb and 55 Mb, respectively. Subsequent analysis disclosed variations in the organization of genome assembly and virulence-associated genes, specifically *Secreted in Xylem* (*SIX*) genes. Hence, these findings not only shed light on the genomic intricacies of Malaysian *Foc* isolates, but also contribute significantly to a deeper understanding of their characteristics. This newfound knowledge holds the potential to play a pivotal role in the development of effective management techniques for Fusarium wilt in our country and beyond. The implications extend to enhancing global efforts in combating this pathogen and advancing the field of Fusarium wilt management.

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