

**COLD PLASMA TREATMENT TO IMPROVE BANANA
GROWTH AND DISEASE TOLERANCE AGAINST
FUSARIUM WILT**

PRIYA A/P RAJAKUMAR

**FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR**

2024

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GROWTH AND DISEASE TOLERANCE AGAINST
FUSARIUM WILT**

PRIYA A/P RAJAKUMAR

**DISSERTATION SUBMITTED IN FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF MASTER
OF SCIENCE**

**INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR**

2024

UNIVERSITI MALAYA

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Name of Candidate: **PRIYA A/P RAJAKUMAR**

Matric No: **17110736/2**

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**COLD PLASMA TREATMENT TO IMPROVE BANANA GROWTH AND
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COLD PLASMA TREATMENT TO IMPROVE BANANA GROWTH AND DISEASE TOLERANCE AGAINST FUSARIUM WILT

ABSTRACT

Banana (*Musa* spp.) is a valuable fruit crop due to its high nutrition and availability throughout the year. Being among the top 10 staple food by production, banana serves millions of lives worldwide. However, banana production is threatened by a destructive disease called Fusarium wilt, caused by the hemi-biotrophic soil-borne pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Developing disease-tolerant banana varieties through conventional breeding or genetic engineering is complicated by the parthenocarpic nature of bananas, which lack seeds for traditional breeding methods. An alternative to these methods is using an eco-agricultural technology called cold plasma (CP) treatment. CP treatment is a non-ionizing radiation that uses electricity and reactive carrier gases (nitrogen, oxygen, helium) to inactivate enzymes, microbial decontamination and for food preservation. Besides, CP treatment also plays a role in crop improvement via growth and disease tolerance improvement. For consistency, the term ‘cold plasma’ will be generally referred as ‘plasma’ throughout the thesis. This study aimed to investigate the plasma effects on the growth performances and Fusarium wilt disease tolerance in banana plants. In this study, tissue culture derived ‘Berangan’ banana plantlets were treated with plasma followed by two months acclimatization in the greenhouse. Growth performances in terms of height, leaf number and stem diameter were measured and evaluated over a three-month duration for the plasma-treated plants in comparison with the untreated plants. Plasma treatment exhibited a favourable impact on plant growth, with no observed negative effects. In addition, a bioassay experiment was conducted by inoculating both plasma-treated and untreated plants with *Foc* Tropical Race 4 (*Foc* TR4) to study the plasma effect on *Foc* tolerance. Controls were also prepared (non-*Foc* inoculated plants comprises of plasma-treated and untreated). Disease

severity index (DSI) and disease progression were recorded throughout five weeks post-*Foc* inoculation. Plasma-treated plants displayed delayed disease progression at week four compared to the untreated plants that started exhibiting severe wilting symptoms in week three. Nevertheless, the plasma-treated plants were found to be highly susceptible to *Foc* infection just like the untreated (non-plasma). However, DSI values in terms of leaf symptom index (LSI) and rhizome discoloration index (RDI) for the plasma-treated plants were slightly lesser (LSI: 4.0, RDI: 6.0) compared to the untreated plants (LSI: 4.2, RDI: 6.6). To understand the regulatory mechanism underlying CP treatment which influences the growth and defence in banana plants, several *Foc*-responsive genes involved in defence and growth were selected for this study including defence-related (*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*) and growth-related (*Cytochrome P450*, *NAC68*, *CAT*) genes. qPCR analysis of root samples collected at 2-, 24-, 48- and 72-hours post-inoculation (hpi) revealed differential expression of those genes following CP treatment and *Foc* TR4 inoculation. CP treatment was found to differentially induce the defence and growth genes, particularly in conjunction with *Foc* TR4 infection. The findings not only shed light on the potential use of CP to increase yield by improving growth and tolerance against Fusarium wilt but offer insights into the possible mechanisms contributing to the improved traits.

Keywords: Banana, *Musa* sp., cold plasma, plant improvement, gene expression.

RAWATAN PLASMA SEJUK UNTUK MENINGKATKAN PERTUMBUHAN DAN DAYA KETAHANAN PISANG TERHADAP PENYAKIT LAYU

FUSARIUM

ABSTRAK

Pisang (*Musa spp.*) merupakan tanaman buah-buahan yang berharga disebabkan oleh kandungan nutrisinya yang tinggi dan ketersediaannya sepanjang tahun. Tersenarai dalam 10 makanan utama berdasarkan pengeluaran, pisang menyumbang kepada jutaan manusia di seluruh dunia. Walau bagaimanapun, produksi pisang menghadapi ancaman serius oleh penyakit layu Fusarium yang disebabkan oleh sejenis patogen hemi-biotropik tanah iaitu '*Fusarium oxysporum* f. sp. *cubense* (Foc)'. Penghasilan varieti toleran melalui pembiakan tanaman atau kejuruteraan genetik adalah sangat mencabar kerana pisang merupakan tanaman partenokarpik yang tidak mempunyai benih. Alternatif kepada kaedah-kaedah ini adalah penggunaan teknologi eko-pertanian iaitu rawatan plasma sejuk. Rawatan plasma merupakan radiasi tidak mengion yang menggunakan tenaga elektrik dan gas pembawa reaktif (nitrogen, oksigen, helium) untuk pentakaktifan enzim, membasmi mikroorganisma, dan memelihara makanan. Tambahan pula, rawatan plasma turut memberi sumbangan dalam penambahbaikan tanaman melalui peningkatan pertumbuhan dan toleransi terhadap penyakit. Bagi konsistensi istilah 'plasma sejuk' akan dirujuk sebagai 'plasma' sepanjang tesis. Kajian ini bertujuan untuk mengkaji kesan plasma terhadap prestasi pertumbuhan dan ketahanan penyakit layu Fusarium dalam pokok pisang. Dalam kajian ini, anak pokok pisang Berangan daripada kultur tisu dirawat dengan plasma dan kemudiannya diaklimatisasi selama dua bulan di rumah hijau. Prestasi pertumbuhan dinilai dari segi ketinggian, bilangan daun dan diameter batang, selama tiga bulan bagi pokok-pokok yang dirawat dan tidak dirawat dengan plasma. Rawatan plasma telah menunjukkan impak yang baik terhadap pertumbuhan tumbuhan, tanpa kesan negatif yang diperhatikan. Tambahan pula, kajian bioasai dijalankan dengan

menginokulasikan kumpulan pokok yang dirawat dan tidak dirawat plasma menggunakan patogen '*Foc* Tropical Race 4 (cold *Foc* TR4)' untuk mengkaji kesan plasma terhadap toleransi '*Foc*'. Kawalan (pokok tidak diinokulasi dengan '*Foc*' terdiri daripada pokok dirawat dan tidak dirawat dengan plasma) juga disediakan. Indeks keterukan penyakit dan perkembangan penyakit dianalisis dan diperhatikan sepanjang lima minggu selepas inokulasi '*Foc*'. Pokok yang dirawat dengan plasma menunjukkan perkembangan penyakit yang lewat pada minggu ke-empat berbanding dengan pokok yang tidak dirawat yang mula menunjukkan gejala layu yang teruk pada minggu ke-tiga. Pokok yang dirawat dengan plasma didapati sangat mudah dijangkiti oleh jangkitan '*Foc*' seperti pokok yang tidak dirawat. Walau bagaimanapun, nilai keparahan penyakit dari segi indeks simptom daun (ISD) dan indeks pewarnaan rizom (IPR) bagi pokok yang dirawat dengan plasma lebih rendah (ISD: 4.0, IPR: 6.0) berbanding dengan pokok yang tidak dirawat (ISD: 4.2, IPR: 6.6). Bagi memahami mekanisme plasma yang mempengaruhi pertumbuhan dan pertahanan dalam pokok pisang, beberapa gen respons awal '*Foc*' yang terlibat dalam pertahanan dan pertumbuhan telah dipilih, termasuk, gen respon pertahanan ('*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*') dan gene respon pertumbuhan ('*Cytochrome P450*, *NAC68*, *CAT*'). Profil ekspresi gen yang dianalisis menggunakan sampel akar yang dikumpulkan pada 2, 24, 48, dan 72 jam selepas inokulasi menunjukkan perbezaan ekspresi selepas rawatan plasma dan inokulasi '*Foc* TR4'. Rawatan plasma didapati secara berbeza menggalakkan gen pertahanan dan pertumbuhan, terutamanya dengan asosiasi jangkitan '*Foc* TR4'. Dapatan daripada kajian ini dapat membantu untuk menilai potensi penggunaan plasma pada pisang bagi meningkatkan hasil dengan meningkatkan pertumbuhan dan daya tahan terhadap penyakit layu *Fusarium* serta memahami mekanisme yang menyumbang kepada ciri-ciri yang diperbaiki.

Kata Kunci: Pisang, *Musa* sp., plasma sejuk, peningkatan tanaman, ekspresi gen.

ACKNOWLEDGEMENTS

First and foremost, praises and thanks to the almighty God for His blessings and for giving me the strength to complete my research project successfully. Next, I would like to thank my guardian angels in heaven, my grandma (Nallammah) and grandpa (Kuppusamy) for showering their blessings. I would like to express my sincere gratitude to my main research supervisor, Dr Nur Ardiyana Rejab (Universiti Malaya) and co-supervisor Dr Nadiya Akmal Baharum (Universiti Putra Malaysia) for their constant support, immense knowledge as well as for giving me a whole new experience in conducting research on banana plants under their supervision.

Special thanks to Dr. Syarifah Aisyafaznim Sayed Abdul Rahman and Dr. Fiqri Dizar Khaidizar, Dr Najiah Mohd Sadali and Associate Proffesor Dr. Muhamad Shakirin bin Mispan for their guidance and support in completing my lab works. I am very much thankful to my seniors, Arulthevan and Hazel Marie for providing me extra knowledge on my research project. Heartfelt thanks to my fellow lab mates; Ahmad Husaini, Nur Fatin Husna, Ong Sheue Ni, Nur Hikmah, Zuha Zarrin, Siti Aminah, Zakariya and Afiqah Insyirah (UPM) for all those best memories and their constant support throughout my postgraduate journey.

I am extending my sincere thanks to my friends: Christina Jane, Nishanthinie, Kausalyaa, Dhivyadharshini, Thamendaran, Vealesh, Jeyssen George and Keshindran for their support and motivation along my research journey. I am extremely grateful to my family: Rajakumar, Sarasvathy, Dr Ranjini, Dr Priyanka, Angammah, Marrayee, Muhunthan, Suganthi, Saarvin, Varshaa, Maniam, Letchumy and Thirisha for their unconditional love, financial support and sacrifices along my research journey. Lastly, I would like to thank all my other family members, friends, juniors, seniors, lab assistants, final year project students who were there to support me in the completion of my research.

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LIST OF SYMBOLS AND ABBREVIATIONS

β	:	Beta
%	:	Percent
$^{\circ}\text{C}$	:	Degree Celsius
μg	:	Microgram
μM	:	Micromolar
μL	:	Microlitre
cm	:	Centimetre
g	:	Gram
h	:	Hour
kHz	:	Kilohertz
kV	:	Kilovolts
L	:	Litre
M	:	Molar
mA	:	Milliampere
mg	:	Milligram
min	:	Minute
mL	:	Millilitre
mM	:	Millimolar
N_2	:	Nitrogen
ng	:	Nanogram
nm	:	Nanomolar
O_2	:	Oxygen
OH^-	:	Hydroxide ions
pH	:	Potential of hydrogen

<i>psi</i>	:	Pound per square inch
<i>S</i>	:	Svedberg
<i>sec</i>	:	Second
<i>V</i>	:	Volt
AC	:	Alternating current
AGE	:	Agarose gel electrophoresis
Blast	:	Basic local alignment search tool
Blastn	:	Nucleotide blast
bp	:	Base pair
CAT	:	Catalase
cDNA	:	Complementary deoxyribonucleic acid
CEBiP	:	Chitin elicitor binding protein
ChiH	:	Chitinase
CP	:	Cold plasma
Ct	:	Cycle threshold
CTAB	:	Cetyl Trimethylammonium Bromide
Cytochrome P450	:	Cytochrome P450 714B3-like
DC	:	Direct current
DEPC	:	Diethyl pyrocarbonate
DH	:	Double haploid
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTPs	:	Deoxynucleotide triphosphates
DSI	:	Disease severity index
EDTA	:	Ethylenediaminetetraacetic acid

EtBr	:	Ethidium bromide
FAO	:	Food and Agriculture Organization
<i>Foc</i>	:	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i>
H ₂ O	:	Water
H ₂ O ₂	:	Hydrogen peroxide
hpi	:	Hours post-inoculation
IPR	:	Indeks simptom rizom
ISD	:	Indeks simptom daun
JA	:	Jasmonic acid
LiCl	:	Lithium chloride
LSI	:	Leaf symptom index
MAMP	:	Microbe-associated molecular pattern
MS	:	Murashige & Skoog
MTI	:	MAMP-triggered immunity
MW	:	Microwave
NAC68	:	NAC domain containing protein 68-like
NaCl	:	Sodium chloride
NCBI	:	National Centre for Biotechnology Information
PAHO	:	Pan American Health Organization
PAL	:	Phenylalanine ammonia lyase
PAMP	:	Pathogen-associated molecular pattern
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
POD	:	Peroxidase

PPO	:	Polyphenol oxidase
PR	:	Pathogenesis-related
PR1	:	Pathogenesis-related 1
PRR	:	Pattern-recognition receptor
PTI	:	PAMP-triggered immunity
PVP	:	Polyvinylpyrrolidone
qPCR	:	Quantitative polymerase chain reaction
R	:	Race
RDI	:	Rhizome discolouration index
RF	:	Radio frequency
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
rpm	:	Revolution per minute
RPS2	:	Ribosomal protein S2
rRNA	:	Ribosomal RNA
RT-qPCR	:	Real-time polymerase chain reaction
SA	:	Salicylic acid
SAR	:	Systemic acquired resistance
SMS	:	Sequence manipulation site
STR4	:	Subtropical race 4
TBE	:	Tris-borate-EDTA
TF	:	Transcription factor
T _m	:	Melting temperature

TR4	:	Tropical race 4
Tris-HCl	:	Tris-Hydrochloride
UV	:	Ultraviolet
VCG	:	Vegetative compatibility groups
w/v	:	Weight by volume
WHO	:	World Health Organization
WRKY1	:	WRKY transcription factor 1
WRKY22	:	WRKY transcription factor 22
WRKY50	:	WRKY transcription factor 50

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CHAPTER 1: INTRODUCTION

1.1 Research background

Banana (*Musa* spp.) is a tropical cash crop being grown in 130 countries (Alzate Acevedo *et al.*, 2021) and is vital for food security (Ye *et al.*, 2020). It is well known as the world's most important fruit crop due to its production volume and trade (Dita *et al.*, 2018). Banana is the second most produced fruit following citrus, contributing to nearly 16 % of the world's fruit production and it is the fourth most important food crop following rice, wheat and corn (Alzate Acevedo *et al.*, 2021; Namuddu, 2022). Banana's high nutritive value categorizes it as one of the paramount fruit crops globally (Dong *et al.*, 2020), making it among the top 10 staple foods worldwide (Dita *et al.*, 2018). Despite its prominence, banana plants are facing serious threat by a destructive disease called Fusarium wilt. Fusarium wilt is a fungus pathogen attack caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Dita *et al.*, 2018). Fusarium wilt has led to considerable loss in banana production since the 18th century after being reported first in Java, Indonesia among the Gros Michel banana strain (Raman *et al.*, 2021). Differential host and pathogenicity determine the three physiological races of *Foc*. They are *Foc* R1, *Foc* R2, and *Foc* R4, in which *Foc* R1 and *Foc* R4 are the most important races that have led to greater yield loss. *Foc* R1-resistant cultivar, Cavendish has been dominating banana production and export since 1960s (Dong *et al.*, 2020). Half of the banana production relies mainly on the soma clones derived from a single triploid genotype, Cavendish. However, the emergence of tropical race 4 (*Foc* TR4) was a serious threat to Cavendish of genome group AAA because Cavendish varieties were susceptible to *Foc* TR4 despite the environmental conditions (Thangavelu *et al.*, 2020). This is because *Foc* TR4 are capable of infecting Cavendish varieties under both stressed and non-stressed conditions (both tropical and subtropical regions) (Liu *et al.*, 2020). This has led to serious concern about the deficit in banana production and export, which seriously threatens food security.

The spread of this fungal pathogen is via root-to-root contact, infected planted materials (rhizomes, suckers), soil attached to planting materials, irrigation, flood, workers, vehicles, footwears and unsterilized potting compost (Thangavelu *et al.*, 2019). Long-term efficient control of Fusarium wilt is still challenging and it involves the development of novel control measures (Ismaila *et al.*, 2022). Control measures such as crop rotation, fumigation, fungicides and microbial antagonists are being applied to bring Fusarium wilt disease under control. However, these control measures are not practical due to time consumption, labour consuming, environmental pollution and high economic cost (Dita *et al.*, 2018; Tang *et al.*, 2020). Besides, negative impacts free chemicals to control diseases are still not available in tropical countries (Ismaila *et al.*, 2022). Genetic engineering or plants transformation has been deployed to develop *Foc*-resistant or tolerant cultivar (Wang *et al.*, 2021). Nevertheless, genetic manipulation in plants may take a long time to produce the final materials ready to be planted in the field (Ahmad and Mukhtar, 2017). Thus, developing *Foc*-resistant cultivars are important to control Fusarium wilt and to increase banana production worldwide.

Thus, a relative-faster and more convenient eco agricultural method, cold plasma treatment (CP), which is a non-ionizing radiation has gained attention. Plasma is the fourth state of matter which is being produced because of energy induction into mixture of gases involving gas ionization and formation of active components such as ultraviolet radiation (UV). CP has exhibited promising capabilities in stimulating plant growth and enhancing disease tolerance. Notably, recent research on Hemp (*Cannabis sativa* L.) reported that CP up-regulated WRKY1 transcription factor and genes responsible for cannabinoids production that involved in plant defence (Iranbakhsh *et al.*, 2020). Additionally, CP has been demonstrated to increase resistance against *Ralstonia solanacearum* (causing bacterial wilt disease) in tomato (Jiang *et al.*, 2014) and increase yield in wheat (Jiang *et al.*, 2014). Unlike traditional control methods, CP offers a quicker

and more environmentally friendly solution as an alternative to produce superior plant materials.

However, despite its promising applications across various crops, the utilization of CP treatment to enhance growth and disease tolerance against Fusarium wilt in banana plants has not yet been investigated. Therefore, this study aimed to evaluate the effect of CP treatment based on the growth performances and Fusarium wilt (*Foc* TR4) disease tolerance in banana plants. In this study, plasma-treated banana plants were evaluated for growth performances by observing their growth parameters such as height, number of leaf and stem diameter. Then disease tolerance was evaluated by observing and recording their disease severity and disease progression following *Foc* TR4 inoculation. Subsequently, gene expression analysis of three growth and six defence-related genes were carried out via quantitative PCR (qPCR) to understand the gene regulation by plasma which provides an insight on the mechanism underlying CP treatment.

1.2 Problem statement

Fusarium wilt of banana, referred to as Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most devastating diseases which possess serious threat to banana (*Musa* spp.), the most important fruit crop worldwide. Developing *Foc*-resistant banana cultivars is a major challenge in preventing the loss of banana plants, as well as protecting food security at large. Despite trying plethora of control measures, Fusarium wilt disease could never be brought under control. This is because all the available control measures are either labour-intensive, energy consuming or expensive. Besides, *Foc*-infected banana plants are hard to detect unless symptoms are expressed. Quarantine is so far the best method to prevent the spread of *Foc* to neighbouring banana plants. This is because once the fungus is introduced in the soil, it can survive for decades by producing survival structures such as chlamydospores. Meanwhile, resistant cultivars are still not available. Thus, the development of resistant

banana plants is the main goal of genetic improvement programs. However, since domesticated bananas are parthenocarpic fruit means that the plants are sterile, breeding programs are very challenging to be carried out.

Thus, CP treatment, an emerging technology has attracted the scientific attention in improving disease tolerance and growth performance by upregulating the growth and defence-related gene expression in banana plants. Based on several published reports, CP treatment was found to improve disease tolerance in plants such as tomato by increased production of reactive oxygen species (ROS) and nutrient absorption (Jiang *et al.*, 2014). Besides CP treatment also found to improve growth in plants such as wheat and *Arabidopsis thaliana* through improved germination rate and moisture content which improves plants' readiness to fight against pathogens (Bafoil *et al.*, 2018). However, there are no reports on the successful use of plasma to improve any traits related to growth and disease tolerance in banana. It is important to note that, to date, all plasma related research carried out on plants only reported the use of seeds as starting materials for plasma treatment. This is a first study that utilize plasma on plantlets since cultivated bananas lack seeds and are propagated vegetatively, often through tissue culture methods, no seeds can be produced from the cultivated banana due to sterility.

1.3 Research questions

1. Does the application of cold plasma treatment on banana plants improve their growth performance?
2. Does the application of cold plasma treatment on banana plants improve their tolerance against Fusarium wilt disease?
3. What are the expression patterns of selected growth and defence-related genes in *Foc* inoculated plants treated with plasma in comparison with control group (untreated)?

1.4 Objectives

This study aims:

1. To assess the effects of cold plasma treatment on growth performance of banana plants;
2. To determine the effects of cold plasma treatment on tolerance against fungal pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in banana plants;
3. To determine the gene expression patterns of growth and defence-related genes in *Foc* inoculated plants treated with plasma in comparison with control group (untreated).

1.5 Hypothesis

Cold plasma treatment improves growth performance and reduces the severity of Fusarium wilt disease by upregulating the expression of growth and defence-related genes in banana plants.

CHAPTER 2: LITERATURE REVIEW

2.1 Banana

The term 'banana' refers to both banana and plantain. Banana (*Musa* spp.) which is the fifth most vital horticultural fruit crop is of family Musaceae (Raman *et al.*, 2021; Wang *et al.*, 2021). Banana is a widely grown cash crop in the tropical and subtropical countries which originated from South-East Asia (Ye *et al.*, 2020). Banana plays crucial role in the economy of developing countries and being grown in over 130 countries in the tropics, with global production of 170 million tons annually (FAO-STAT 2021). Banana is being mainly cultivated by smallholder farmers for domestic consumption, local or regional market purposes (Adero *et al.*, 2023).

Banana is valued and ranked fourth among the leading fruit crops (after rice, wheat and corn) due to their starch and high nutrient content (Wang *et al.*, 2021). Thus, banana is well known as the paramount fruit crop worldwide (Dong *et al.*, 2020). The value of banana is determined by their stenothermic characteristics which enables them to fruit throughout the year in diverse environments, as well as flavour and nutrient content (Al-Daour *et al.*, 2020). Banana can be categorized into two major culinary groups: dessert bananas (sweet bananas) and cooking bananas (plantain) which are rich in starch and regularly cooked or processed (Marta *et al.*, 2022).

Large base of genetic diversity in banana was from the natural crosses between *Musa acuminata* (A genome) and *Musa balbisiana* (B genome) which are the origin of the seedless banana varieties. According to the botanical view, genus *Musa* is divided into seminiferous species (inedible fruits) and parthenocarpic varieties (fleshy seedless fruits) which includes *M. acuminata* and *M. balbisiana*, the wild species at the origin of the cultivated varieties (Lescot *et al.*, 2020).

Hybridization between the *Musa* species and subspecies that have diverged in many tropical Southeast regions, islands and western Melanesia produced different banana cultivars. *M. acuminata* species is part of origin of all cultivars except for the Fe'I vitamin-rich bananas (A genome, $2n = 22$). Meanwhile, *M. balbisiana* (B genome, $2n = 22$) is associated with *M. acuminata* in most of the cultivars. On the other hand, *M. textilis* and *M. schizocarpa* have been contributed to only few cultivars. Several subspecies of *M. acuminata* distributed from Northeast India to New Guinea, includes *siamea*, *burmannica*, *burmannicoides*, *malaccensis*, *zebrina* and *banksia*. The current diploids and triploids with reduced fertility are the result of additional hybridization of *M. acuminata* within them or with other *Musa* species (Martin *et al.*, 2020).

Hundreds of banana cultivars are being cultivated worldwide, but Cavendish is the most common dessert banana cultivar being cultivated in large monoculture plantations. Large scale farmers mainly cultivate Cavendish bananas for local and international markets' commercialization (Raman *et al.*, 2021; Adero *et al.*, 2023). Around 47 % of global banana production is made up of Cavendish varieties of which about a third is for export (Viljoen *et al.*, 2020). 'Sukali Ndiizi', 'Gros Michel', 'Rasthali', and 'Lakatan' are other dessert banana cultivars produced in several countries worldwide, where they are sold for economic and social importance. Plantain is widely grown in Central and West Africa. Besides, the East African Highland banana (EAHB) is being cultivated in East Africa, especially in the Great Lakes region of Africa where it serves as a source of livelihood for millions of people (Adero *et al.*, 2023).

India is the largest producer of banana (Raman *et al.*, 2021). Meanwhile, Ecuador is the world's largest banana exporting country with profit reaching approximately \$1 billion (Fung *et al.*, 2019). According to United Nations Food and Agriculture Organization, banana serves as a major source of food to alleviate starvation among populations of poor regions such as Africa (Wang *et al.*, 2021). Both plantain and unripe

banana are consumed after cooking while ripe banana (dessert banana) can be eaten raw. On the other hand, a small portion of bananas undergo industrial processing (Aurore *et al.*, 2009).

All parts of banana have nutritional value and medicinal importance. Banana helps the body to retain nutrition such as calcium, phosphorus and nitrogen for healthy tissues. Besides, banana is a good supplementary staple food as it is low in calories. Nearly, 116 calories of energy are being provided by hundred grams of banana. Two bananas are sufficient to provide energy needed for 90 mins of workout. Bananas are essential part of human diet as they are rich in carbohydrates, vitamins and minerals (Ranjha *et al.*, 2020). Ripe bananas are rich in magnesium and potassium which are essential for controlled muscle contraction (Aurore *et al.*, 2009). Meanwhile, banana plants are also rich in antioxidants and minerals such as iron, which are important for weaning infants and mothers (Mohapatra *et al.*, 2010).

The whole banana plant is very useful in food, pharmaceutical, packaging, feed and industrial applications (Mohapatra *et al.*, 2010). However, banana plants are facing serious threat due to pathogen attacks that causes diseases and lowers banana plant production worldwide (Wang *et al.*, 2021). Triploid nature of banana that is restricted and inflexible genetically is making them more susceptible to pathogen attacks. This is because the large-scale production of banana is from vegetative propagation of selected genotypes (Adero *et al.*, 2023). In the last century, the banana industry has undergone a dramatic loss due to an epidemic known as Fusarium wilt (Wang *et al.*, 2021).

2.2 Fusarium wilt of banana

Panama disease is referred to Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Wang *et al.*, 2021). It is also known as classical vascular wilt disease (Dita *et al.*, 2018). Fusarium wilt is a serious lethal disease which has more recently been intensified by *Foc* tropical race 4 (*Foc* TR4) in Cavendish cultivars (AAA) (Wang *et al.*, 2021). Fusarium wilt has led to serious destruction on export plantations and food security.

2.2.1 The pathogen

Foc is characterized as polyphyletic because it is a heterogeneous fungus which has multiple origins. *Foc* initially maintains biotrophic relationship with the host which later becomes necrotrophic therefore causing the plants to die. Thus, they are referred as hemi biotroph (Dita *et al.*, 2018). The fungus which is responsible for Fusarium wilt of banana is very diverse compared to other *formae speciales* in *Fusarium oxysporum* species. *Foc* has three different physiological races and 24 vegetative compatibility groups (VCGs) which is responsible for diseases that affect genus *Musa* such as *M. acuminata*, *M. balbisiana*, *M. schizocarpa* and *M. textilis* (Mostert *et al.*, 2017; Thangavelu *et al.*, 2019). *Foc* race is determined based on their type of hosts and pathogenicity, namely race 1 (R1), race 2 (R2) and race 4 (R4). Race 1 affects Gros Michel (AAA) and Silk (AAB), race 2 affects the hybrid triploid cultivar cooking bananas (Bluggoe, ABB) and race 4 affects all the cultivars in Cavendish (AAA) varieties in addition to race 1 and race 2 susceptible varieties as it has a broad host range (Dita *et al.*, 2018; Liu *et al.*, 2020).

2.2.2 History of the disease

Fusarium wilt disease was first reported in Java, Indonesia during the 18th century infecting the mostly cultivated strain, Gros Michel and later has spread to many banana growing countries such as India and China (Raman *et al.*, 2021). Fusarium wilt that

affected the Gros Michel banana industry was *Foc* R1. Cavendish cultivars were used to replace the Gros Michel banana as they were resistant to *Foc* R1. However, the later cultivation of Cavendish varieties under seasonal abiotic stresses led them to be susceptible to *Foc* R4 in the subtropical regions. However, after 1989, Cavendish varieties were found to be susceptible to *Foc* R4 in the tropical regions as well (Dita *et al.*, 2018). Thus, *Foc* R4 was further divided into subtropical Race 4 (STR4) and tropical race 4 (TR4). *Foc* STR4 only affects Cavendish varieties in the subtropical regions and *Foc* TR4 affects both in the tropical and subtropical regions. *Foc* TR4 is more virulent compared to *Foc* STR4 as it can infect Cavendish banana varieties under both stressed and non-stressed conditions (Liu *et al.*, 2020).

Epidemic of TR4 started in Taiwan in the 1960s and then expanded to Southeast Asia and China. This epidemic was later found to emerge in the Middle East, Africa and India. Recently, the TR4 epidemic was found to be emerged in Columbia (Garcia- Bastidas *et al.*, 2020).

2.2.3 Pathogen survival and infection process

TR4 is known as a soil-borne fungus capable of reproducing asexually by microconidia, macroconidia and chlamydospores production which are their survival structures. TR4 persists and survive at soil or in host tissue for a long period by producing chlamydospores. Besides, the ability of TR4 to cause disease at low inoculum level by residing at certain depth in soil makes it difficult to manage. Detecting TR4-infected plants is difficult because symptoms only start to display six months post-infection (incubation period). Factors such as environment, inoculum level, and resistance influence the incubation period and delays symptom expression (Pegg *et al.*, 2019). *Foc* infection originates from the secondary and tertiary roots through the vascular pathways. Infection of *Foc* in larger roots occurs via rhizome and pseudostem and remain localized. *Foc* can infect via sites of wound in host plants. For example, wound caused by nematode

penetration creates easy entry for fungus (Dita *et al.*, 2018). They receive nutrients for growth from cell exudates (Pegg *et al.*, 2019). *Foc* infection involves multiple cycles in the host plant tissue (Mon *et al.*, 2021). Living xylem protects the host plants from deeper invasion of pathogen in their rhizome, thus becoming a barrier against advanced pathogen infection. However, in mature vessels of host tissue, pathogen can move passively via the empty lumens (Pegg *et al.*, 2019).

Infection begins at the root and tends to be a slow movement (75 cm in four weeks) while movement of pathogen picks up in the mature xylem vessels (30 cm within two or three days) accompanied by generation of new spores (MacHardy and Beckman, 1981). Thickened hyphae and micro-conidia will be produced and later the hyphae will develop into chlamydospores along the intra-and intercellular spaces (Li *et al.*, 2011). Distribution of pathogen in the pseudostem occurs upon reaching the rhizome within a period of two weeks. This obstructs the mobilization of the nutrients (Li *et al.*, 2017). Infection by *Foc* triggers host defence responses such as gels, tyloses and lignification, leading to immobilization of pathogen. Pathogen can be completely suppressed by tyloses, which later become impregnated by phenolic compounds because of longer gel persistence. In resistant varieties, host defence response is rapidly distributed at different locations such as root base, root, and rootlets (MacHardy and Beckman, 1981). However, in susceptible varieties, water shortage due to vascular plugging after the pathogen has invaded the xylem vessels and rhizome indicates invasion of pathogen (Pegg *et al.*, 2019). This causes reduced transpiration and symptoms expression such as faint pale-yellow streak formation, leaf chlorosis from lower leaves to upper leaves, wilt of leaves and splitting of leaf bases longitudinally (Viljoen *et al.*, 2020). Expression of symptoms due to *Foc* infection is accompanied by release of fusaric acid (Liu *et al.*, 2020).

During the later pathogenesis, pathogens move to the parenchyma and cortex to invade weak plant tissue. Release of chlamydospores and conidia from the degraded host tissue

to the environment occurs when the host tissue collapses (Pegg *et al.*, 2019). Reproductive structures are the main route of *Foc* dispersion. Human activity, contaminated water, animals and wind can lead to the movement of propagules for short or long distances (Dita *et al.*, 2018). Movement of workers between plantations are the major source for pathogen spread to neighbouring plantations (Viljoen *et al.*, 2020).

2.2.4 Control methods for Fusarium wilt

Most of the recommended crop management practices are outside the reach of smallholder farmers as they are knowledge-laden, labour-intensive, and expensive (Tinzaara *et al.*, 2018). Besides, mishandling the very common chemical measure, pesticide has led to severe chronic intoxications (Rodríguez *et al.*, 2017). Based on the report by the Pan American Health Organization (PAHO) and the World Health Organization (WHO), there are three million cases of acute pesticide poisoning. Developed and developing countries were reported to have 0.25 % and 0.5 % of mortality rate due to poisoning, respectively (PAHO, 2002). Thus, minimizing the use of pesticides to reduce the associated risks is being encouraged by The Food and Agriculture Organization of the United Nations (FAO) and the European Union in Directive (2009)/128/EC (FAO, 2017).

Meanwhile, there is no effective way to detect an infected banana plant unless symptoms are expressed. The only effective way to control TR4 infection for now is by quarantining infected and neighbouring banana plants (Pegg *et al.*, 2019). Developing resistant banana cultivars is a major challenge in meeting global customer needs and preventing food insecurities. The selection of resistant cultivars which are still not available is an effective control strategy against the *Foc* TR4 infection and is a main goal of genetic improvement programs (Damodaran *et al.*, 2020). However, since domesticated banana is a parthenocarpic fruit, it is very hard to carry out breeding programs to produce resistant cultivars. Besides, polyploidy, lower reproductive fertility

and complete sterility of bananas are the reasons for lower success rate of breeding programs (Zhizhou *et al.*, 2020).

2.3 Cold plasma (CP)

Cold plasma (CP) treatment is an advanced eco-agricultural technology that has been demonstrated to stimulate the growth of plants. CP treatment is a non-ionizing radiation which refers to ‘cold plasma’ (Jiang *et al.*, 2014). Plasma is defined as a neutral ionized gas. Plasma which is the result of energy induction into mixture of gases is known as the fourth and highest energy state of matter after solids, liquids and gases (Yodpitak *et al.*, 2019).

Variety of gases are being employed in the process of plasma generation such as argon, helium or their combination with oxygen (Sruthi *et al.*, 2022). This induction process involves gas ionization and active components formation such as charged particles (OH^- , H_2O^+ , electrons), reactive oxygen species (ROS), reactive nitrogen species (RNS), excited molecules (excited O_2 , N_2), radicals, positive ions, negative ions and ultraviolet radiation (UV) (Sruthi *et al.*, 2022). The formation of CP is from microwave (MW), radio frequency (RF), direct current (DC), and alternating current (AC). CP is used in different setups such as dielectric barrier, jet plasma and corona discharges (Bagheri *et al.*, 2020).



Figure 2.1: Spark of plasma generated from plasma jet device (adapted from Joh *et al.*, 2014).

2.3.1 Cold plasma (CP) and agriculture applications

CP is an emerging technology that has wide applications in agriculture (Attri *et al.*, 2020). One of the applications is in post-harvest aspect. This technology can be used for microbial inactivation by reactive oxygen species (ROS) and nitrogen species (Bagheri *et al.*, 2020). Thus, CP treatment is effective in storage and increasing shelf life of banana. Worldwide, nearly 1.4 million bananas are being wasted daily due to mould growth. CP treatment disinfects the storage atmosphere of bananas without affecting their quality, thus resulting in no spoilage of bananas compared to controls that does not undergo plasma treatment. In this case, CP treatment is an important antimicrobial treatment which enhances the shelf life of bananas (Trivedi *et al.*, 2019).

In addition, CP has also been used to inactivate enzymes responsible for browning, discoloration and flavour deterioration which improves the quality of banana (Gu *et al.*, 2021). CP has successfully been applied to plant seeds which improved their wetting properties, germination rate and yield. Seeds that have been treated with CP were found to gain extended shelf-life (Yodpitak *et al.*, 2019). CP treatment was proven to improve yield in oilseed rape by increasing their permeability, seed germination, plant growth and capacity to uptake water (Ling *et al.*, 2018). CP is effective in upregulating the vitality of seeds without the occurrence of any gene mutations (Jiang *et al.*, 2014). Plants that have been treated with CP have shown improved resistant towards biotic and abiotic stress. Due to its significant effect on the growth of plants, it is an effective way to develop plants that are disease resistant. CP treatment is used to sterilize seeds, which kills cells of bacteria or spores of few *Bacilli* spp. UV radiation and radicals from plasma are used for direct killing and inactivation of microbes (Jiang *et al.*, 2014).

Based on a study carried out using wheat, plasma treatment significantly improved chlorophyll and moisture content of wheat, as well as physiological level of wheat, both

of which led to improved growth of wheat (Jiafeng *et al.*, 2014). Based on a study carried out using *Arabidopsis thaliana*, plasma treatment was found to increase their germination rate (Bafoil *et al.*, 2018).

Moreover, CP treatment activates endogenous substances in seeds, rejuvenate seeds, promotes plant growth and increases yield of plants. Based on previous study with tomato, CP treated tomato plants were observed to have increased germination potential and germination rate compared to control plants without plasma treatment. The height, thickness of leaves, diameter of stem, and dry weight of plasma-treated tomato were also found to be better than of control plants without plasma treatment (Jiang *et al.*, 2014). CP treatment also improves the uptake of phosphorus and nitrogen in tomato plants by the accumulation of root and shoot biomass. This improves the root activity, length, surface area and volume of the root. Thus, CP is an alternative way to improve nutrient absorption in tomato (Jiang *et al.*, 2018). Nutrient is an important factor which plays an effective role for both plant growth and prevention of disease (Jiang *et al.*, 2014).

Plasma-treated plants took more days to wilt following inoculation by *Ralstonia solanacearum*. On the other hand, plasma untreated controls wilted faster following inoculation. This is because the progress of disease in plasma-treated plants was slower compared to in untreated plants. The disease severity observed in plasma-treated tomato plants was only 75 % even 20 days after inoculation while untreated plants were observed to have 100 % disease severity. Thus, this study showed that the growing status of plants influences the resistance against disease by affecting their readiness to fight pathogenic infection (Jiang *et al.*, 2014).

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) which plays a role during the early stages of pathogen infection. It is very important for plants to develop resistance against pathogen attack. H_2O_2 concentration in tomato tissue that have

undergone plasma treatment rose faster following *R. solanacearum* inoculation in first 24 hours, thus reducing disease severity. Enzymes such as peroxidase (POD) that detoxifies ROS, polyphenol oxidase (PPO) that catalyzes oxygen-dependent phenols oxidation, and phenylalanine ammonia lyase (PAL) that mediates plant salicylic-acid mediated pathogen defence, play an important role in resistance of plants against pathogens, biotic and abiotic stress. All these enzymes significantly increased following *R. solanacearum* inoculation in plasma-treated tomato plants (Jiang *et al.*, 2014).

All the studies above emphasize that plasma treatment has tremendous potentials in improving growth and disease control, thus improving plant varieties. However, they did not highlight the gene regulation, which is a crucial aspect in understanding the CP effects and mechanisms. At the same time, there is no study available on the application of CP treatment on banana plants which can improve their disease tolerance especially against Fusarium wilt which is a major threat to banana plantations.

2.4 Plant genes related to Fusarium infection and plant growth

Host responses against Fusarium wilt in the pathosystem are crucial to be studied for the development of effective and better control measure to overcome Fusarium wilt. There are several defence-related genes (*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*) and growth-related genes (*Cytochrome P450*, *NAC68*, *CAT*) found to be associated with *Foc* infection. These genes are crucial to be studied in terms of their expression for better understanding on the mechanism underlying CP that may improve the growth and disease tolerant trait.

2.4.1 Pathogenesis-related (PR) gene

Accumulation of pathogenesis-related (PR) protein is one of the main characteristics of plant response against biological or abiotic stress. PR protein is important in plant innate immunity and is one of the most abundant from this family of proteins (Niu *et al.*,

2018). Among the 17 families, PR1 protein is the dominant group which is being induced by salicylic acid and pathogen. Therefore, PR1 protein is used as the molecular marker for detecting pathogen-induced systemic acquired resistance (SAR) (Rocha *et al.*, 2022).

Pathogen attack causes pathogen recognition receptors (PRR) to recognize the pathogen-associated molecular patterns (PAMPs) of the pathogen which activates MAMP-triggered immunity (MTI). As a result, various downstream defence responses were activated such as release of defence-related genes which includes *PR* genes (Mohd-Yusuf *et al.*, 2019). Differential expression of *PR1* gene was observed in susceptible and resistant banana against *Foc* R4. *PR1* gene was found to be upregulated in the susceptible cultivar and downregulated in the resistant cultivar following *Foc* TR4 infection (Niu *et al.*, 2018). A study by Zhang *et al.* (2019) reported that *PR1* gene was highly expressed in both resistant and susceptible banana genotypes following *Foc* TR4 infection.

2.4.2 WRKY transcription factors (TFs)

The stress response machinery of the defence relies on TFs. WRKY TFs are established regulators of defence genes (Niu *et al.*, 2018). The WRKY superfamily TFs are the seventh largest in flowering plants and it serves as a promising candidate responsible for plant breeding. This is due to their rigid regulations involving link of WRKYs to forward promoters and specific recognition (Rocha *et al.*, 2022). WRKY TFs modulate the expression of genes responsible for pathogen-associated molecular pattern (PAMP) triggered immunity and effector-triggered immunity. In general, WRKY TFs function as activators or repressors to control gene expression by binding the TTGAC(C/T) W-box cis-element in the promoter region of target genes (Kaliyappan *et al.*, 2016).

It was reported that WRKY TFs regulate seed germination and secondary metabolism (Kaliyappan *et al.*, 2016). In addition to regulating defence genes expression, WRKY TFs also involve in the regulation of disease response pathways regulated by jasmonate and

salicylate. *WRKY* genes are also reported to be highly expressed in wild diploid banana even without *Foc* inoculation suggesting that they might be associated with constitutive defence mechanism (Zhang *et al.*, 2019). *WRKY22* gene displayed higher relative expression level at all hours in BRS Platina cultivar that was inoculated with *Foc* R1 suggesting the incompatible interaction between resistant cultivar and *Foc* R1. Niu *et al.* (2018) also reported the upregulation of *WRKY50* gene during *Foc* infection in banana plants. Thus, this result shows that *WRKY22* and *WRKY50* genes are involved in the plant defence response against *Foc* infection.

2.4.3 Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is the first key enzyme of phenylpropanoid pathway. Monolignol biosynthesis which is derived from phenylalanine via the phenylpropanoid pathway is one of the production mechanisms of lignin. Deposition of lignin which forms the cell wall serves as mechanical barrier against invading pathogens (Mohd-Yusuf *et al.*, 2019). *PAL* gene modulates resistance in the defence responses against pathogen infection in most higher plants. *PAL* gene was found to be upregulated upon *Foc* TR4 infection in 'Berangan' banana plants (Munusamy *et al.*, 2019). Besides, *PAL* gene was also found to be highly expressed in banana genotypes that has improved tolerance against *Foc* TR4 after being treated with endophytic bacteria (Candra *et al.*, 2022).

2.4.4 Chitin elicitor binding protein (CEBiP)

CEBiP protein is responsible for the activation of PAMP-triggered immunity (PTI) which recognizes chitin oligosaccharides (De Jonge *et al.*, 2010). Chitin, an important structural component of fungal cell wall function as a pathogen-associated molecular pattern (PAMP) such as bacterial flagellin. PAMP involved in the induced immune response to inhibit further pathogen colonization and spread (Jian *et al.*, 2019). PTI provides first-hand plant immunity by recognizing exogenous or endogenous elicitors

which are known as pattern-recognition receptors (PRRs) upon pathogen infection. Upon recognizing defence response such as oxidative burst (generation of ROS), strengthening of cell wall and mitogen activated protein kinase cascades are activated. Besides, PRRs also serves as microbe-associated molecular pattern (MAMP), which then activate MTI. *CEBiP* gene was found to be upregulated upon *Foc* TR4 infection in resistant cultivars (Das *et al.*, 2023).

2.4.5 Putative Chitinase (*ChiH*) gene

Chitin is a well-known component of the fungal cell wall. Pathogen attack induces the production of chitinase enzyme which functions in inhibiting the growth of fungal pathogens in vitro by degrading chitin. Besides, chitinase enzyme also accumulates around the fungal hyphae in vivo. Damage caused by fungal pathogen is believed to be reduced by the help of chitinase overexpression. *ChiH* gene was upregulated in both resistant and susceptible banana genotypes upon *Foc* TR4 infection (Zhang *et al.*, 2019). According to another study carried out in transgenic banana, overexpression of chitinase is correlated with *Foc* race 1 resistance (Subramaniam *et al.*, 2006).

2.4.6 Cytochrome P450 protein

Cytochrome P450 family genes participate in the hormone homeostasis and regulates the plant growth (He *et al.*, 2019). Cytochrome P450 is also known for its involvement in detoxification of oxygen free radicals actively released during stressful conditions such as pathogen attack which sustain the plant growth. Besides, cytochrome P450 also helps Cavendish bananas during their ripening stage by causing accumulation of proteins in the skin and pulp of matured fruits (Gamez *et al.*, 2019). Besides, cytochrome P450 is also involved in the jasmonic acid (JA) pathway (Dong *et al.*, 2020). JA is an endogenous growth-regulator which regulates important growth and developmental processes such as inducing stomatal opening and transporting glucose (Ruan *et al.*, 2019). Cytochrome

P450s also involve in the synthesis of lignin and other defence compounds such as isoflavonoids, hydroxamic acids and glucosinolates in banana. Upon infection by *Foc* TR4, *Cytochrome P450* gene was upregulated in both resistant and susceptible banana genotypes suggesting its role in defence mechanism (Zhang *et al.*, 2019; Dong *et al.*, 2020).

2.4.7 NAC domain containing protein 68-like (NAC68)

NAC domain containing genes produces plant-specific TFs and it is one of the largest TF families which has multiple roles in stress regulation (biotic and abiotic), plant development, fruit maturation and hormone signal transduction pathways (Na *et al.*, 2016). According to Niu *et al.* (2018), the *NAC68* gene is responsible in determining the resistance of banana against *Foc*. NAC TFs were found to be overexpressed in egg plant (*Solanum melongena*) which suppress their resistance against bacterial wilt (Na *et al.*, 2016). NAC TFs were also found to suppress leaf senescence which promotes grain yield (Zhao *et al.*, 2015).

2.4.8 Catalase (CAT)

Catalase (CAT) is an antioxidant enzyme which serves role as ROS (H_2O_2 , O_2^- , and free radicals) scavenger to maintain plant homeostasis (Thye *et al.*, 2022). ROS causes oxidative stress which can lead to mitochondrial dysfunction and cell injury by oxidizing lipids, nucleic acids and amino acids. This affects various physiological activities in plants such as disruption of membrane via lipid peroxidation, programmed cell death, senescence, stunted growth in plants (Thye *et al.*, 2022). *CAT* gene was found to be upregulated within 96 hours post-*Foc* infection in banana plant. CAT detoxifies ROS and minimizes the damage to the host cell by degrading ROS into water and oxygen and sustain plant development (Mohd-Yusuf *et al.*, 2019).

CHAPTER 3: METHODOLOGY

3.1 Plant material

Rooted stage of tissue culture-derived banana plantlets of *Musa acuminata* cv. 'Berangan' was obtained from Plant Biotechnology Incubator Unit (PBIU), Universiti Malaya. Healthy banana plantlets with green leaves (~ 5 leaves), height and stem diameter of 7 cm and 0.5 cm, respectively were selected for this study. Banana plantlets were carefully removed from Murashige & Skoog (MS) agar and rinsed using tap water prior to CP treatment. In total, there were 80 plantlets that were divided into several treatment groups as listed below:

- (1) Group 1: treated (plasma) + *Foc* inoculated (20 plants)
- (2) Group 2: untreated (non-plasma) + *Foc* inoculated (20 plants)
- (3) Group 3: treated (plasma) + non-*Foc* inoculated (20 plants)
- (4) Group 4: untreated (non-plasma) + non-*Foc* inoculated (20 plants)

3.2 Cold plasma (CP) treatment

Banana plantlets of group 1 and 3 were subjected to CP treatment that was conducted at the Department of Physics, Universiti Malaya and will henceforth be denoted as 'treated'. Conversely, plantlets that did not undergo plasma treatment were referred as 'untreated'. A non-thermal plasma jet (Figure 3.1) was employed for the banana plantlets' treatment with CP. The plasma jet was generated via a pin electrode housed in a quartz tube and ground ring electrode. The discharge was produced under atmospheric air at 15 kV, 20 kHz for 15 secs and targeted at corm of the banana plantlets. The plasma treatment was carried out by the research collaborator at Plasma Technology Research Centre, Department of Physics, UM.

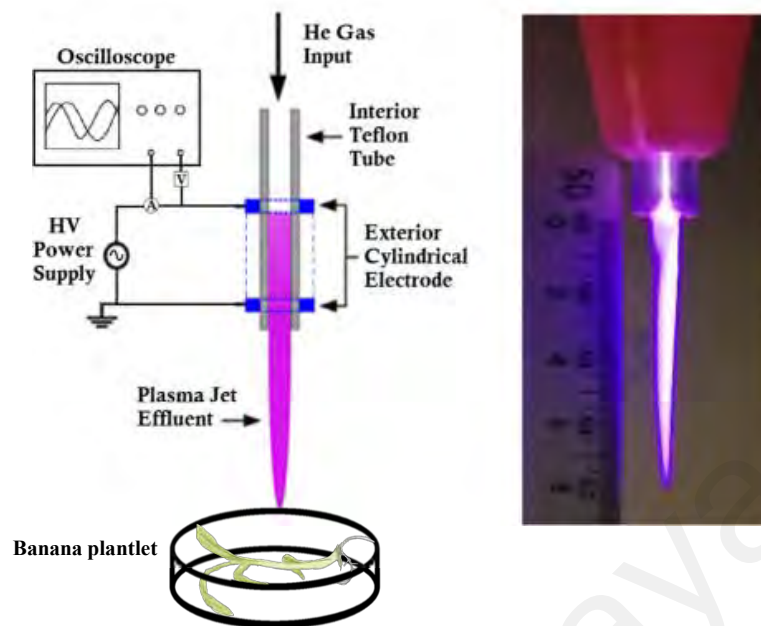


Figure 3.1: Experimental setup of plasma jet device (adapted from Auguste *et al.*, 2023).

3.3 Plantlets acclimatisation

Plasma-treated and untreated banana plantlets were maintained in hydroponic system supplied with AB medium (UH Agropionics, Malaysia) for two months. The AB medium is a commercial fertilizer designed for hydroponic cultivation conditions that contains essential nutrients such as macronutrients (nitrogen, phosphorus, potassium), micronutrients (iron, manganese, zinc, copper) and other trace elements required for optimal plant growth and development. This hydroponic procedure was done to acclimatize the plantlets and allow more roots to form and to reach minimum size suitable for further analysis including the bioassay experiment. Rooted plants were then transferred to polybag containing black soil, cocopeat, and compost in the ratio of 10: 5: 3. Plants were placed in a shaded area and sprayed regularly with tap water. Plasma-treated and untreated plants were then transferred to Plant Biotech Facility (PBF), Universiti Malaya with controlled temperature of 25 ± 2 °C and 12-hour photoperiod following average natural light exposure in Malaysia. Plants were watered regularly every

two to three days and fertilized once a week with N: P: K (15: 15: 15) fertilizer (GardenWell). Plants were then transferred to individual pots after 14 days.

3.4 Fungal isolate and verification

Foc TR4 isolate was obtained from co-supervisor Dr. Nadiya Akmal Baharum (Universiti Putra Malaysia, UPM). This fungal isolate originated from an infected *M. acuminata* cv. ‘Cavendish’s inner stem in Kuala Terengganu, Terengganu, Malaysia. The *Foc* race verification was conducted by co-supervisor’s team from UPM.

Taq PCR Master Mix (2X, red dye) (Bio Basic, Canada) was used to verify the identity of the *Foc* isolate using DNA derived from *Foc* isolate subcultured on PDA as template via polymerase chain reaction (PCR). The DNA was isolated earlier from the *Foc* isolate using Biospin Fungus Genomic DNA extraction kit (Bioer Technology, China) according to manufacturer’s instructions. The PCR reactions’ components were assembled based on Table 3.1. PCR and amplification were conducted using PCR thermal cycler (ThermoFisher Scientific, USA) based on the PCR condition showed in Table 3.2. Two different primer pairs were used in this study which were *Foc* 1/ *Foc* 2 and TR4. The sequence of both forward and reverse primers used in this PCR experiment are shown in Table 3.3.

Table 3.1: PCR reaction components used.

Component	Volume (μL)	Final concentration
<i>Taq</i> PCR master mix	12.5	1×
10 μM forward primer	1	0.4 μM
10 μM reverse primer	1	0.4 μM
Template cDNA	0.5	< 1000 ng
Nuclease-free water	10	-
Total	25	

Table 3.2: PCR condition.

Step	Temperature (°C)	Time	Cycles
Pre-denaturation	94	4 mins	1
Initial denaturation	94	30 secs	30-35
Annealing temperature	60	30 secs	
Extension	72	1 min	
Final Extension	72	10 mins	1
Hold	4	-	-

Table 3.3: Primers used in this study.

Primers	Sequences	Annealing temperature	Reference
<i>Foc</i> 1/ <i>Foc</i> 2_F	5'- CAG GGG ATG TAT GAG GAG GCT- 3'	60 °C	Lin <i>et al.</i> , (2008)
<i>Foc</i> 1/ <i>Foc</i> 2_R	5'— GTG ACA GCG TCG TCT AGT TCC- 3'	60 °C	Lin <i>et al.</i> , (2008)
TR4_F	5' – CAC GTT TAA GGT GCC ATG AGA G- 3'	60 °C	Dita <i>et al.</i> , (2010)
TR4_R	5' – CGC ACG CCA GGA CTG CCT CGT GA- 3'	60 °C	Dita <i>et al.</i> , (2010)

The *Foc* TR4 isolate used in this study was maintained in 4 % water agar which was prepared by melting 40 g of gelrite powder (Duchefa Biochemie, Netherlands) in 1 L of slightly boiled sterile distilled water. The agar was then let to solidify at room temperature (26 ± 2 °C) following preparation prior to subculture. The isolate was then sub-cultured on Potato Dextrose agar (PDA) (Oxoid, England) which was prepared by suspending 39 g of agar powder in 1 L of sterile distilled water. Prepared PDA media were autoclaved at 15 psi, 121 °C for 15 mins following preparation. The sub-cultured isolate was then incubated in dark condition at room temperature (26 ± 2 °C) for eight days prior usage.

3.5 Preparation of *Foc* TR4 suspension culture

The fully grown *Foc* TR4 mycelia on PDA were cut into five to ten pieces of approximately 1 cm × 1 cm plug using sterile scalpel and inoculated into 1 L of Potato Dextrose Broth (PDB) (Oxoid, England). PDB was prepared by suspending 24 g broth powder in 1 L of sterile distilled water. The prepared PDB media were autoclaved at 15 psi, 121 °C for 15 mins following preparation. The inoculated suspension culture was incubated at room temperature (26 ± 2 °C) and was manually swirled twice a day. On the 7th day post-inoculation, spore concentration was determined using haemocytometer (Weber, England) under microscope (Leica, Germany). This was done by transferring 10 µL of *Foc* TR4 suspension culture to haemocytometer using pipette and covered using microscope cover slip. Final concentration of suspension culture was adjusted to 1×10^6 spores/mL using sterile distilled water (Mohd-Yusuf *et al.*, 2019).

3.6 Fusarium wilt bioassay

Plants of group 1 (treated (plasma) + *Foc* inoculated) and group 2 (untreated (non-plasma) + *Foc* inoculated) were subjected to Fusarium wilt bioassay using a double-tray method according to protocol by Mohd-Yusuf *et al.* (2019). Healthy plants of two months old, with ~ 5 leaves, white roots with length of minimum 5.0 cm, and stem with diameter of 0.5 cm to 1.0 cm were selected for Fusarium wilt bioassay. The plants were carefully uprooted and soil were removed without damaging their roots. Prior to inoculation, roots of plants were rinsed with tap water to remove any soil residues. Then, plants were soaked into the *Foc* TR4 suspension culture (1×10^6 spores/mL) for two hours before re-planting into their original soil and were labelled accordingly. The plants were then transferred into a double-container apparatus containing a smaller tray with holes (43 × 29 × 9 cm) that fits into a bigger tray at the bottom (46 × 31 × 20 cm). On the other hand, plants of

group 3 and 4 (non-*Foc* inoculated) were soaked with sterile distilled water and serve as controls. All plants were continued to be watered and fertilized as stated in section 3.3.

3.7 Observation of plant growth parameters

Observation of plant growth parameters was done for four plants both from group 3 and 4 (non-*Foc* inoculated plants) for a duration of three months starting from day-1 of plasma treatment. Growth parameters such as plant height, number of leaves and stem diameter were observed and recorded. Data was presented as percentage of increment based on the readings measured on day 1 (plasma treatment) and day 90.

3.8 Disease progression observation

Response of both *Foc* inoculated (Group 1 and 2) and non-inoculated plants (Group 3 and 4), towards *Foc* infection was observed from 1st week till 5th week post-inoculation. The number of yellowing and wilting leaves was recorded for each group. In each group, difference in disease progression indicated by clear yellowing of leaves between plasma-treated and untreated plants were recorded.

3.9 Disease scoring using leaf symptom index (LSI) and rhizome discolouration index (RDI)

Final evaluation of plants involved in section 3. 8 was carried out at the end of 5th week post-inoculation based on Mohd-Yusuf *et al.* (2019). Number of wilting and yellowing leaves were observed and recorded. Then, all the plants were carefully uprooted from soil. After that, vertical dissection of their rhizomes was done for internal symptoms observation and to record the various level of brownish discolorations. Plants' responses against Fusarium wilt were determined based on leaf symptoms index (LSI) and rhizome discoloration index (RDI) based on LSI and RDI scores showed in Table 3.4 (Mak *et al.*,

2004). Then, disease severity index (DSI) was calculated for both LSI and RDI from the scores obtained using the formula:

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

Then, the DSI values were translated into four designations such as resistant, tolerant, susceptible, and highly susceptible referring to guidelines stated in Mak *et al.* (2004). If the LSI and RDI is tolerant and susceptible respectively, the cultivar will be considered susceptible. If the LSI and RDI is tolerant and resistant respectively, the cultivar will be considered tolerant. Only when both LSI and RDI scores fall in resistant category, the cultivar will be considered as a resistant cultivar.

Table 3.4: Translation of DSI scales (Mak *et al.*, 2004).

DSI scales for LSI	DSI scales for RDI	Translation
1	1	Resistant
Between 1.1 and 2	Between 1.1 and 3	Tolerant
Between 2.1 and 3	Between 3.1 and 5	Susceptible
Between 3.1 and 4	Between 5.1 and 8	Highly susceptible

3.10 Gene selection and primer design for RT-qPCR

To investigate the possible mechanisms through which plasma may contribute to better tolerance to Fusarium wilt disease, several genes related to defence and growth were selected. In general, these genes were found to play an important role in banana defence against *Foc* as reviewed in section 2.4. Six defence-related genes including *PRI* (Van den Berg *et al.*, 2007), *WRKY22* (Bai *et al.*, 2013), *WRKY50* (Niu *et al.*, 2018), *PAL* (Bai *et al.*, 2013), *CEBiP* (Bai *et al.*, 2013), *ChiH* (Dong *et al.*, 2019) and three growth-related genes which are *Cytochrome P450 714B3-like* (Gamez *et al.*, 2019), *NAC68* (Niu *et al.*,

2018), *CAT* (Mohd-Yusuf *et al.*, 2019) were selected to study their expression profile following *Foc* inoculation. Full name of the genes is listed in Appendix A. Primers for all the above defence and growth-related genes were designed based on DH Pahang genome sequences from Banana Genome Hub database (<https://banana-genome-hub.southgreen.fr>) and Phytozome (<https://phytozome-next.jgi.doe.gov>) using Primer 3 software (<https://primer3.ut.ee>). Designed primers were then evaluated using PCR Primer Stats software (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). All the designed primers are listed in Table 4.2 and 4.3 in section 4.6. Ribosomal protein S2 (*RPS2*) gene was used as housekeeping gene in this study and their primer sequences were obtained from Chaurasia *et al.*, (2016). Table 3.5 shows the primer sequence of the reference gene, *RPS2*.

Table 3.5: Reference gene used for quantitative real-time PCR (sequence obtained from Chaurasia *et al.*, (2016).

Primer	Primer sequences	Size (bp)	Tm (°C)	Product size (bp)
<i>RPS2_F</i>	CGGGATTGCCTGATATTGTG	20	53.4	200
<i>RPS2_R</i>	CCCTCACAAATTGCGGATAC	20	53.9	

3.11 Validation of primers for gene expression analysis

3.11.1 Polymerase chain reaction (PCR)

Taq PCR Master Mix (2X, red dye) (Bio Basic, Canada) was used to validate the functionality of the designed primers using DNA extracted from roots of *Foc* infected ‘Berangan’ banana plants as template via polymerase chain reaction (PCR). This template DNA was extracted earlier using Qiagen DNA extraction kit (Qiagen, Germany) according to manufacturer’s description. Root tissue samples were homogenized using mortar and pestle and up to 100 mg of powder were transferred to a 1.5 mL

microcentrifuge tube. Then 400 μ L buffer AP1 and 4 μ L RNase were added followed by vortexing and incubation for 10 mins at 65 °C. The microcentrifuge tube was inverted two to three times during incubation. Next, 130 μ L of buffer P3 were added, mixed and incubated for 5 mins on ice. The lysate was then centrifuged for 5 mins at 13,000 rpm and pipetted into a QIAshredder spin column placed in a 2 mL collection tube followed by centrifugation for 2 mins at 13,000 rpm.

The flow-through was transferred into a new 1.5 mL microcentrifuge tube without disturbing the pellet. Then, 1.5 volumes of buffer AW1 were added and mixed by pipetting. Next, 650 μ L of the mixture were transferred into a DNeasy Mini spin column placed in a 2 mL collection tube followed by centrifugation for 1 min at 10,000 rpm. The flow-through was discarded. The spin column was placed into a new 2 mL collection tube and 500 μ L buffer AW2 was added followed by centrifugation for 1 min at 10,000 rpm. The flow-through was discarded. Then, another 500 μ L of buffer AW2 were added and followed by centrifugation for 2 mins at 13,000 rpm. The spin column was transferred to a new 1.5 mL microcentrifuge tube and 100 μ L buffer AE were added for elution followed by incubation for 5 mins at room temperature. Subsequently, centrifugation was done for 1 min at 10,000 rpm.

The PCR reactions components were assembled based on Table 3.6. PCR and amplification were conducted using PCR thermal cycler (ThermoFisher Scientific, USA) based on the PCR condition showed in Table 3.7.

Table 3.6: PCR reaction components used.

Component	25 μ L reaction	Final concentration
<i>Taq</i> PCR master mix	12.5	1 $\times$
10 μ M forward primer	1	0.4 μ M
10 μ M reverse primer	1	0.4 μ M
Template DNA	0.5	< 1000 ng
Nuclease-free water	10	-

Table 3.7: PCR condition.

Step	Temperature ($^{\circ}$ C)	Time	Cycles
Pre-denaturation	94	4 mins	1
Initial denaturation	94	30 secs	30-35
Annealing temperature	60	30 secs	
Extension	72	1 min	
Final Extension	72	10 mins	1
Hold	4	-	-

3.11.2 Agarose gel electrophoresis (AGE) for PCR products

The PCR products were separated via AGE using 1.0 % (w/v) agarose gel which was prepared by melting 0.2 g of agarose powder (Promega, Spain) in 20 mL of 1 $\times$ TBE buffer (8.0 pH). The 1 $\times$ TBE buffer was prepared by making a 1:10 dilution on the 10 $\times$ TBE buffer prepared by dissolving 108.0 g Tris-Base (Merck, Germany), 55.0 g boric acid (Chemiz, Malaysia). The pH of the 1 $\times$ TBE buffer was adjusted to 8.0 before being subjected to autoclave at 15 psi, 121 $^{\circ}$ C for 15 mins. Approximately 1 μ L of GelStarTM Nucleic Acid gel stain (Sigma-Aldrich, USA) was added into the molten agarose for

nucleic acid staining. The molten agarose was allowed to solidify in room temperature for approximately 40 mins. The samples were then migrated in the 1.0 % (w/v) agarose gel with 1 × TBE buffer (pH 8) at 120 V, 400 mA for 23 mins.

After separation, the resulting DNA fragments in the stained agarose gel was visualized and analysed using high performance UV transilluminator (ThermoFisher Scientific, USA). The gel image was captured using Gel imaging system (Perkin Elmer Geliance 200, USA). 100bp DNA Marker (Bioteke, Malaysia) was used as marker to identify the approximate size of amplified DNA fragments on the agarose gel.

3.11.3 Purification of DNA fragments from agarose gel

Elution of DNA fragments was done using FavorPrep™ Gel/ PCR purification kit (Favorgen, USA) according to the manufacturer's protocol. The desired DNA fragments of approximately 100 - 200 bp was excised by cutting the agarose gel surrounding the desired DNA fragments using a clean scalpel. The excised gel up to 300 mg was transferred to a 1.5 mL microcentrifuge tube. Then, 500 µL of FADF buffer were added to the microcentrifuge tube and mixed well by vortexing. After that, the microcentrifuge tube was subjected to incubation at 55 °C for 10 mins and interval vortexing was done to accelerate the dissolving of the excised gel. The sample mixture was let to cool down at room temperature. Next, a FADF column was placed into a collection tube and 800 µL of sample mixture was transferred into the column and subjected to centrifuge at 13,000 rpm for 30 secs. The flow-through was then discarded. Subsequently, 750 µL of wash buffer (ethanol added) were added to the FADF column and subjected to centrifuge at 13,000 rpm for 30 secs. The flow-through was discarded. The FADF column was then again centrifuged at full speed for an additional 3 mins for column matrix drying. Then, the dried FADF column was placed into a new 1.5 mL microcentrifuge tube followed by addition of 40 µL of elution buffer to the membrane centre. The FADF column was

incubated at room temperature for 1 min and subjected to centrifuge at full speed for one minute to elute the DNA.

The concentration and the purity of the purified DNA fragments were measured using NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, USA). Finally, the purified DNAs were then outsourced for sequencing.

3.11.4 DNA sequencing and sequence analysis

Purified DNAs were sent for sequencing at 1st Base company (Malaysia). The quality of the sequenced target genes was visualized and evaluated using BioEdit software (version 7.2). Subsequently, both forward and reverse sequences were aligned using BioEdit software (version 7.2). The aligned sequences were blasted against banana genome (*Musa acuminata*) using BLASTN program using Phytozome (<https://phytozome-next.jgi.doe.gov>) and NCBI database to confirm the identity of the target genes.

3.12 RNA extraction

Tissue samples were collected from roots of all four groups of plants mentioned in 3.1 at four different time points which are at 2-, 24-, 48- and 72-hours post-inoculation (hpi), respectively. For each time point, three replicates were collected. Samples were homogenized using mortar, pestle and liquid nitrogen before being kept at - 80 °C until further processing. Total RNA extraction was done using Cetyl Trimethylammonium Bromide (CTAB) method according to Kistner & Matamoros (2005). CTAB extraction buffer was prepared by dissolving 2 % CTAB powder (Sigma-Aldrich, USA), 2 % of polyvinylpyrrolidone powder (PVP) (Bio Basic, Canada), 0.1 M of Tris-HCl (pH 8), 25 mM ethylenediaminetetraacetic acid (EDTA) and 2 M sodium chloride (NaCl) and 2 % of β -mercaptoethanol (Biomax, Malaysia) in 1.5 mL of diethyl pyrocarbonate (DEPC) (Bio Basic, Canada) water.

The CTAB extraction buffer was incubated at 65 °C for 5 mins and fast cool centrifuged. Around 150 – 200 mg of homogenized samples were transferred into pre-cooled 1.5 mL microcentrifuge tube. Then, 600 µL of CTAB buffer were added to the sample and incubated at 55 °C for 5 mins. Next, 600 µL phenol: chloroform: isoamyl alcohol, 25: 24: 1 (PCI) (Axon Scientific, Malaysia) was added and incubated at 55 °C for 5 mins followed by centrifugation at 13,000 rpm for 10 mins at 4 °C. The upper phase was collected and transferred into a new 1.5 mL microcentrifuge tube. Then, 1 volume of PC1 was added and incubated at 55 °C for 5 mins followed by centrifugation at 13,000 rpm for 10 mins at 4 °C. The upper phase was collected and transferred into a new 1.5 mL microcentrifuge tube. Next 1/3 volume of 8 M LiCl₂ were added to the collected upper phase and incubated at 4 °C overnight. Then, the incubated (overnight) sample was centrifuged at 13,000 rpm for 30 mins at 4 °C. The supernatant was removed. The pellet was then washed using 1 mL of 2 M LiCl₂ and centrifuged at 13,000 rpm for 5 mins at 4 °C. Then, the pellet washed twice using 70 % ethanol (grade of molecular biology) (Merck, Germany) and centrifuged at 13,000 rpm for 5 mins at 4 °C after each wash. Centrifugation was done again at 13,000 rpm for 3 mins at 4 °C and the supernatant was removed. The pellet was then dissolved by adding 30 µL of RNase-free water. The extracted RNA samples were then stored at - 80 °C for downstream applications.

3.13 DNase treatment

All the extracted RNA samples were then subjected to DNase Treatment using DNase I (RNase-free) kit (NewEngland BioLabs, USA), for the removal of genomic DNA according to manufacturer's description. The DNase treatment reactions components were assembled on ice based on Table 3.8.

Table 3.8: DNase treatment reactions components.

Components	100 μ L reaction
RNA	$\sim 10 \mu\text{g}$
DNase I reaction buffer (10X)	10 μ L (1 $\times$)
DNase I (RNase-free)	1 μ L (2 units)
Nuclease-free water	to 100 μ L

Then, the prepared reaction component mixture was incubated at 37 °C for 10 mins. Next, 1 μ L of 0.5 M EDTA was added followed by heat inactivation at 75 °C for 10 mins. Subsequently, RNA quantification was carried out to check the concentration and purity of the RNA using the NanoDrop 2000 spectrophotometer (ThermoScientific, U.S.A.). The absorbance value of the RNA was checked at wavelengths of 230 nm (A230), 260 nm (A260), and 280 nm (A280).

3.14 Agarose gel electrophoresis (AGE) for RNA samples

The extracted RNA samples were mixed with 6 $\times$ loading dye (Fermentas International Inc., Canada) in the ratio of 5: 1. Then, the loading dye-RNA mix were separated via AGE using 1.0 % (w/v) agarose gel with 1 $\times$ TBE buffer (pH 8, 0.089 M) at 120 V, 400 mA for 23 mins to check their integrity before and after DNase treatment. After separation the resulting RNA fragments in the stained agarose gel was visualized and analysed using

high performance UV transilluminator (ThermoFisher Scientific, USA). The gel image was captured using Gel Imaging System (Perkin Elmer Geliance 200, USA).

3.15 cDNA synthesis

Total RNA extracted from all samples were converted to cDNA using Viva cDNA synthesis kit (Vivantis, Malaysia) according to manufacturer's description. RNA-primer mixture was prepared as shown in Table 3.9. The RNA-primer mixture was incubated at 65 °C for 5 mins and chilled on ice for 2 mins. Then, the mixture was spin down. Then, cDNA synthesis mix was prepared as indicated in Table 3.10. The 10 µL of cDNA mixture was added into the RNA-primer mixture and mixed gently followed by brief centrifugation. The mixture was incubated at 42 °C for 60 mins. The reaction was then terminated by incubating the mixture at 85 °C for 5 mins followed by chilling on ice and brief centrifugation. Then, the synthesized cDNA was verified via PCR using housekeeping gene, *RPS2* followed by AGE. The PCR reactions components were assembled based on Table 3.11. PCR and amplification were conducted using PCR thermal cycler (ThermoFisher Scientific, USA) based on the PCR condition showed in Table 3.12. Then, the verified cDNA was used directly for downstream applications.

Table 3.9: RNA-primer mixture.

Components	Volume/ Amount
RNA	0.5 µg - 10 µg
Oligo d(T)	1 µL
10 mM dNTPs mix	1 µL
Nuclease-free water	Top up 10 µL

Table 3.10: cDNA synthesis mixture.

Components	Volume
10X buffer M-MuLV	2 μ L
M-MuLV reverse transcriptase	0.5 μ L
Nuclease-free water	Top up to 10 μ L

Table 3.11: PCR reaction components used.

Component	25 μ L reaction	Final concentration
<i>Taq</i> PCR master mix	12.5	1 $\times$
10 μ M forward primer	1	0.4 μ M
10 μ M reverse primer	1	0.4 μ M
Template cDNA	0.5	< 1000 ng
Nuclease-free water	10	-

Table 3.12: PCR condition.

Step	Temperature ($^{\circ}$ C)	Time	Cycles
Pre-denaturation	94	4 mins	1
Initial denaturation	94	30 secs	30-35
Annealing temperature	60	30 secs	
Extension	72	1 min	
Final Extension	72	10 mins	1
Hold	4	-	-

3.16 Quantitative real-time polymerase chain reaction (RT-qPCR)

Primer efficiency test and gene expression analysis of all the selected defence and growth-related genes were carried out on all samples using real-time qPCR analysis (Applied Biosystems ViiA 7 real time system, ThermoFisher Scientific, USA). In this analysis, gene specific primers of all the target genes described in section 2.4 were used. Real-time analysis was done using the qPCRBIO SyGreen Blue Mix Separate-ROX kit (Biomax Scientific Sdn Bhd.).

The qPCR mastermix was prepared using reactions components assembled in Table 3.13. The qPCR SyGreen Mx was briefly vortexed before the experiment. qPCR was carried out based on the qPCR condition showed in Table 3.14.

Table 3.13: qPCR components used.

Component	Volume (μ L)	Final concentration
qPCRBIO master mix	10	1×
Forward primer	0.8	0.4 μ M
Reverse primer	0.8	0.4 μ M
Template cDNA	1	< 1000 ng
Nuclease-free water	7.4	

Table 3.14: qPCR condition.

Step	Temperature ($^{\circ}$ C)	Time	Cycles
Polymerase activation	95	2 mins	1
Denaturation	95	5 secs	40
Anneal/ Extension	60-65	30 secs	

3.16.1 Primer efficiency test

For determining primer efficiency for a primer, first, template cDNA derived from roots of *Foc* infected 'Berangan' banana plants was diluted through a series of 1: 1 covering four dilution points (dilution 1, 2, 3 and 4). qPCR master mix that is sufficient for 10 reactions was prepared according to Table 3.13. This accommodates nine reactions (two reactions for each dilution point + negative control) and pipetting error. Then the qPCR master mix prepared were loaded into the 96 well plate. For negative control, 1 μ L of nuclease free water was added replacing the template cDNA. These steps were repeated for all the primers for target genes and reference genes. Then, melt curve analysis was done once the run was completed to ensure that the primer pair used is specific for gene expression study. PCR primer efficiency was calculated by first calculating the average Ct values of technical replicates for each dilution. Then, log value of each sample dilution was calculated followed by generating slope of the regression between the log values and the average Ct values calculated. After that, PCR primer efficiency was calculated using the slope value obtained using the formula:

$$\text{Primer efficiency: } (10^{\frac{-1}{\text{slope}}} - 1) \times 100 \quad (3.2)$$

3.16.2 Gene expression analysis

The gene expression analysis was performed for three technical replicates from the pool of three biological replicates. *RPS2* gene was used as the reference gene for this analysis to normalize the qPCR data (Chaurasia *et al.*, 2016). Relative expression levels of all the target genes were calculated via the Ct value using $2^{-\Delta\Delta C_t}$ formula derived from Pfaffl method (Pfaffl, 2007). Then, all the triplicated data were subjected to statistical analysis and normality test. All the raw data were subjected to two-way analysis of variance (ANOVA) followed by Tukey's test (post-hoc). Statistical Package for Social Sciences (SPSS) software was used for all the analysis at the level of 5 %.

CHAPTER 4: RESULTS

4.1 Observation of the *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) growth

Pale violet and pale magenta mycelium of *Foc* TR4 isolate was distributed sparsely on Potato Dextrose Agar (PDA) following eight days of incubation in dark condition at room temperature (26 ± 2 °C) (Figure 4.1). Extensive mycelium growth and turbidity were observed in the Potato Dextrose Broth (PDB) inoculated with *Foc* TR4 mycelium following seven days of incubation in dark condition at room temperature (26 ± 2 °C). The characteristics of macroconidia produced by the culture was either straight or slightly curved with oval and kidney-shaped microconidia under microscopic observation. All the above-mentioned characteristics of *Foc* TR4 isolate were in accordance with Leslie and Summerell (2006). The *Foc* race verification was conducted by our collaborator's team from Universiti Putra Malaysia.

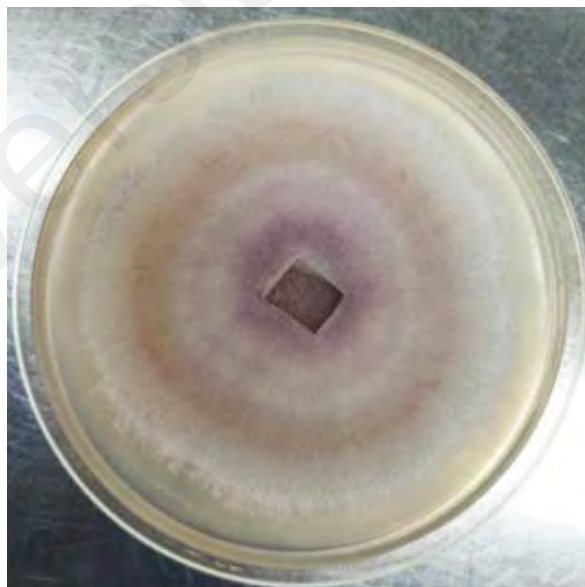


Figure 4.1: Fully grown *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc* TR4) isolate after eight days of incubation on PDA.

4.2 *Foc* isolate verification

Foc verification was carried out by collaborator's team (Dr Nadiya Akmal Baharum, UPM, Malaysia) which verified that the fungal isolate used in this study was *Foc* TR4. High quality DNA with the desired purity range ($A_{260}/A_{280} \sim 1.8$ to 2.0) was successfully isolated from the fungal isolate prior to race verification (Figure 4.2). *Foc* 1/ *Foc* 2 primer pair successfully detected race 4 isolates (Lin *et al.*, 2008) and TR4 primer pair detected more specific identity which is the Tropical race 4 isolates (Dita *et al.*, 2010).

A 242-bp size fragment was produced by PCR from *Foc* gDNA using *Foc* 1/ *Foc* 2 primer pair at annealing temperature of 60 °C (Figure 4.3a). This band showed that *Foc* isolate used in this study is race 4 (*Foc* R4) as reported by Lin *et al.* (2008). PCR amplification using TR4 primer set from Dita *et al.* (2010) produced band around 400 bp at 60 °C (Figure 4.3b). Thus, these results specified the identity of *Foc* isolate used in this study as tropical race 4 (TR4) isolate as reported by Dita *et al.* (2010) based on the presence of a 400 bp-band upon amplification using the TR4 specific primer pair.

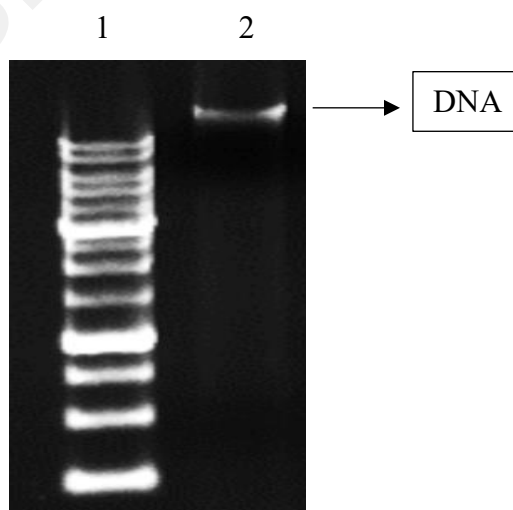
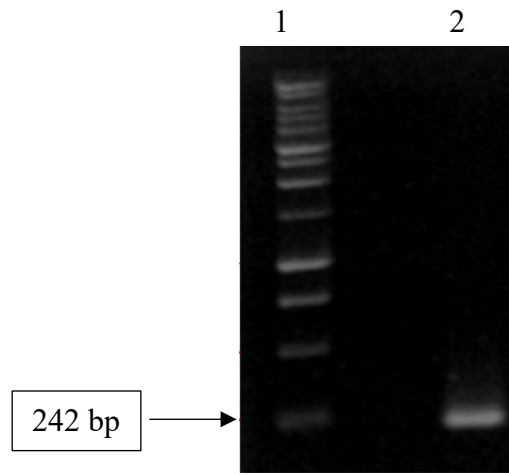
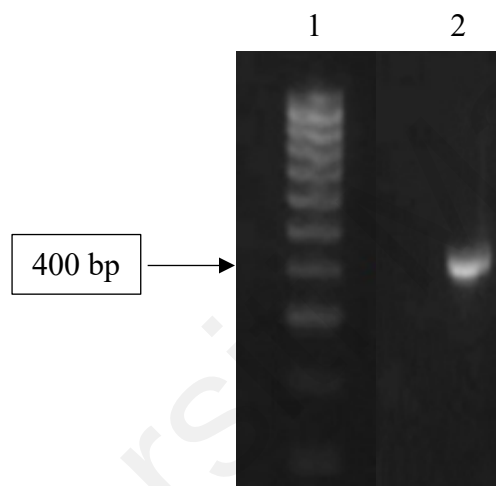


Figure 4.2: The AGE results showing the successful isolation of DNA (Lane 2). Amplicon size was estimated using 1 kb DNA ladder (Lane 1).



(a) *Foc* R4

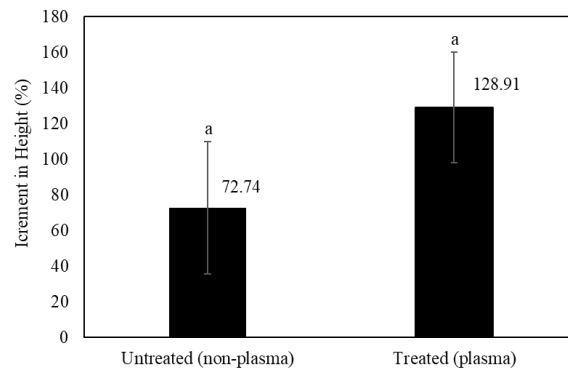


(b) *Foc* TR4

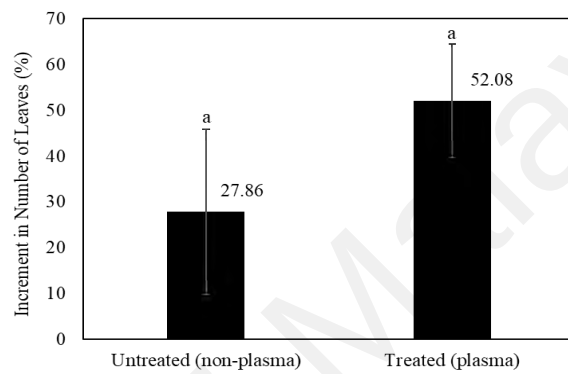
Figure 4.3: Validation of *Foc* race group using *Foc* 1/ *Foc* 2 primer pair and TR4 primer pair. Amplified product at 242 bp (a) and 400 bp (b) in-length verified *Foc* R4 and *Foc* TR4 respectively (Lane 2). Amplicon size was estimated using 1 kb DNA ladder (Lane 1).

4.3 The effect of plasma treatment on the growth of *M. acuminata* cv. 'Berangan'

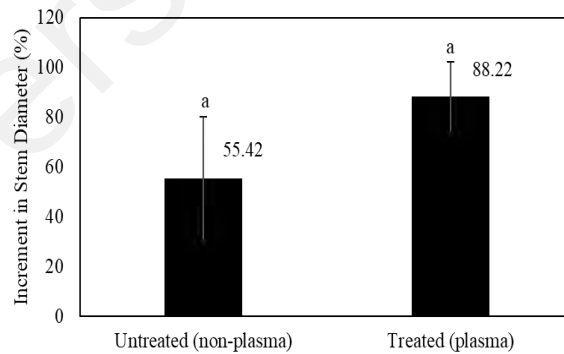
The effect of plasma on the growth of banana cv. 'Berangan' plants was evaluated based on the measurement of three growth parameters which are plant height, leaf number and stem diameter of non-*Foc* inoculated plants, both plasma-treated (Group 3) and untreated (Group 4) for a duration of three months starting from day-1 of plasma treatment. Figure 4.4 shows the average percentage of increment of both the plasma-treated and untreated plants from groups 3 and 4, respectively observed for the duration of three months. In general, values for the growth in terms of height, leaf number and stem diameter were higher in the plasma-treated (Group 3) compared to untreated (Group 4) plants. Plasma-treated plants had 128.91 % increment in height compared to untreated plants that showed 72.74 % increment. For the second trait, plasma-treated plants showed 52.08 % increment in the number of leaves produced compared to 27.86 % increment in untreated plants. Next, for the third parameter, stem diameter, it was found that plasma-treated plants had 88.22 % increment compared to 55.42 % increment in untreated plants. While it is apparent that plasma treatment had a favourable effect on plant growth, the outcomes did not attain statistical significance. Nonetheless, it's important to note that the plasma's impact on plant growth is at least neutral or even potentially beneficial.



(a)



(b)



(c)

Figure 4.4: Effect of plasma treatment on the growth of *M. acuminata* cv. 'Berangan'. Percentage of increment in plant height (a), number of leaves (b) and stem diameter (c) observed for the duration of three months. Data represents the mean $\pm$ standard error (n = 4). Same alphabet on top of error bars of mean represents no significant differences as determined by T-test ($p > 0.05$).

4.4 The effect of plasma treatment on the *Fusarium* wilt disease progression in *M. acuminata* cv. 'Berangan'

The effect of plasma treatment was also evaluated based on the response of 'Berangan' plants to plant pathogen, *Foc* TR4. First, the onset of clear external disease symptoms was assessed based on wilting and yellowing of the leaves. Non-*Foc* inoculated plants from both plasma-treated (Group 3) and untreated (Group 4) groups remained healthy with no visible symptoms (no wilting and yellowing) at the end of 5th week post-inoculation (Figure 4.5b & a). Meanwhile, *Foc* inoculated plants exhibited severe wilting symptoms which progressed from light streaking and yellowing of lower leaves to entire leaves. Plasma-treated plants (Group 1) started exhibiting severe wilting symptoms only from 4th week post-inoculation (Figure 4.5d) compared to untreated plants (Group 2) that started to display symptoms as early as week 3 (Figure 4.5c). These observations showed that plasma treatment led to the delayed disease symptoms in *Foc* inoculated 'Berangan' plants.

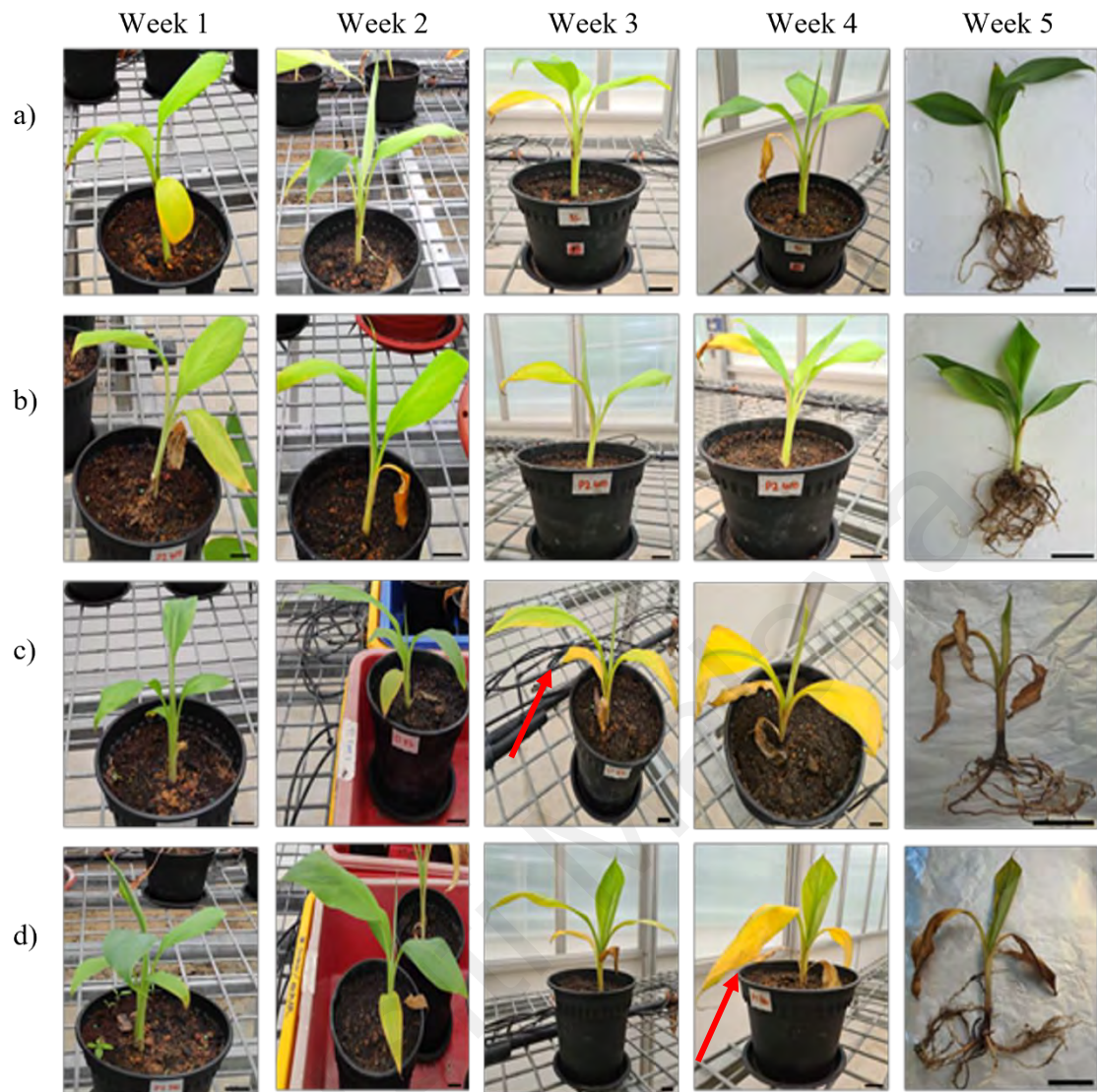


Figure 4.5: Disease progression in plasma-treated and untreated *M. acuminata* cv. 'Berangan' plants following *Foc* TR4 inoculation. Images of representative plants of untreated (non-plasma) + non-*Foc* inoculated (a), treated (plasma) + non-*Foc* inoculated (b), untreated (non-plasma) + *Foc* inoculated (c) and treated (plasma) + *Foc* inoculated (d) groups from week 1, 2, 3, 4 and 5 post-inoculation were shown above. Bars = 5 cm. Red arrows indicate the onset of the symptom.

4.5 Leaf symptom index (LSI) and rhizome discolouration index (RDI)

Next, the response of plasma-treated ‘Berangan’ plants inoculated with *Foc* was also assessed based on disease severity index (DSI) estimated using the LSI and RDI values as mentioned in section 3.9. First, the internal symptoms were evaluated at the end of 5th week post inoculation. No rhizome discoloration was observed at the stellar regions and surrounding tissues of non-*Foc* inoculated plants from both plasma-treated (Group 3) and untreated (Group 4) groups (Figure 4.6b & a, respectively). Meanwhile, *Foc* inoculated plants from both plasma-treated (Group 1) and untreated (Group 2) groups (Figure 4.6d & c) exhibited various level of rhizome discoloration. The observed data indicates that the application of plasma treatment did not confer complete resistance to *Foc* in the plants (Figure 4.6d). Upon closer examination and subsequent scoring using LSI and RDI metrics, it was found that in *Foc* inoculated plants, plasma-treated (Group 1) showed slightly lower LSI and RDI values of 4.0 and 6.0, respectively in comparison with the untreated plants (Group 2) that displayed LSI and RDI values of 4.2 and 6.6, respectively (Table 4.1).

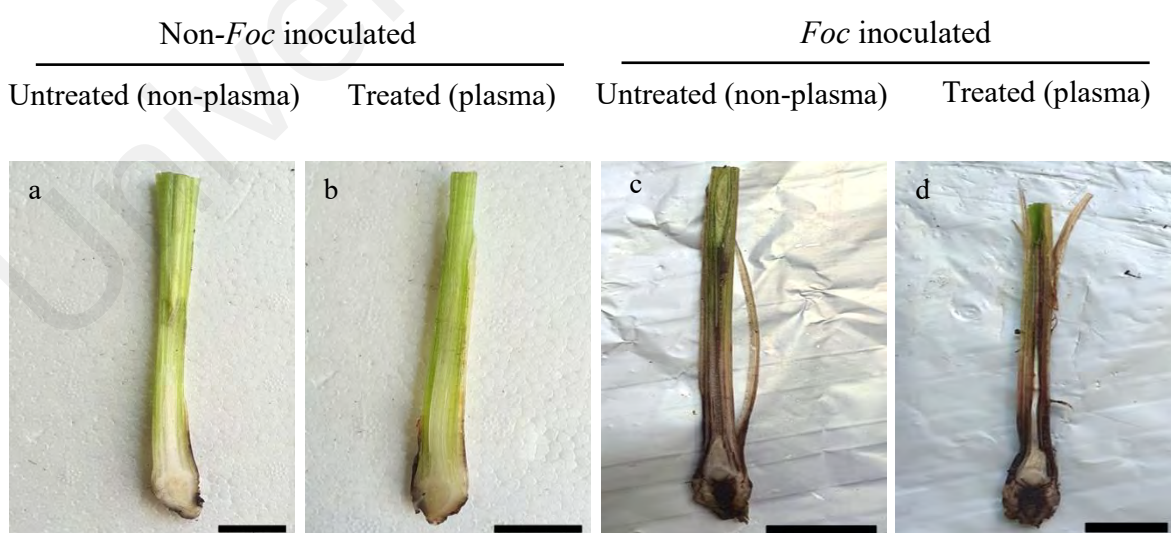


Figure 4.6: Internal disease symptom observation in rhizomes of untreated (non-plasma) + non-*Foc* inoculated (a), treated (plasma) + non-*Foc* inoculated (b), untreated (non-plasma) + *Foc* inoculated (c) and treated (plasma) + *Foc* inoculated (d) *M. acuminata* cv. ‘Berangan’ plants (n≥4). Bars = 2 cm.

Table 4.1: Disease severity index (DSI) showing LSI and RDI from disease scoring of *M. acuminata* cv. 'Berangan' plants (n≥4).

Treatment		DSI scales for LSI	DSI scales for RDI	Translation
<i>Foc</i>	Plasma			
Non-inoculated	Untreated	1.5	1	Tolerant
	Treated	1.5	1	Tolerant
Inoculated	Untreated	4.2	6.6	Highly susceptible
	Treated	4.0	6.0	Highly susceptible

4.6 Primer design and validation

Primers for six defence-related genes including *PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH* and three growth-related genes which are *Cytochrome P450 714B3-like*, *NAC68*, *CAT* were successfully designed and validated by PCR. Results from *in-silico* test done using PCR Primer Stat on the Sequence Manipulation Site website further verified the suitability of the designed primers (Appendix B).

Then, these primers were then used in gene expression study to learn their expression profile following *Foc* TR4 inoculation together with a reference gene, *RPS2*. Table 4.2 and 4.3 show the primer sequences of designed primers for defence and growth-related genes respectively with the expected amplicon size. These primers were validated by PCR. Figure 4.7 shows the successful PCR amplifications indicated by the presence of single bands for all the target and reference genes.

Table 4.2: Primers designed for selected defence-related genes for RT-qPCR.

Primer	Primer sequences (5' – 3')	Size (bp)	Tm (°C)	Product size (bp)
<i>PRI_F</i>	ACCAAACAGCGCATCGG	17	56.4	110
<i>PRI_R</i>	CTTTTGACGGCGTCGATT	18	53.4	
<i>WRKY22_F</i>	GACCCACGATGCTCATT	18	55.0	104
<i>WRKY22_R</i>	TGGGCTTGCTAATGATGAAA	20	52.2	
<i>WRKY50_F</i>	ATGCAGGATCGGGTTCAGAA	20	56.4	101
<i>WRKY50_R</i>	GATTGGGGCTGTTCTTCACC	20	56.2	101
<i>PAL_F</i>	TGAGCCGTTGACGAAGCCGAA	21	61.6	117
<i>PAL_R</i>	TTTGATTTCCTGCTCGATCG	20	55.5	
<i>CEBiP_F</i>	ACACCAGCCTCCCTAGCAGTAT	22	59.8	102
<i>CEBiP_R</i>	GACACTACAACCAGCACTAG	20	55.5	
<i>ChiH_F</i>	TGTACATCGCGGACACCAC	19	60.5	129
<i>ChiH_R</i>	TTCGTACCCGCTCGACATCA	20	58.4	

Table 4.3: Primers designed for selected growth-related genes for RT-qPCR.

Primer	Primer sequences	Size (bp)	Tm (°C)	Product size (bp)
<i>Cytochrome P450_F</i>	TCAATGATTCTATTCTGCTTGATAA	25	50.4	120
<i>Cytochrome P450_R</i>	CATGAAACATCAGGCCCA	18	52.7	
<i>NAC68_F</i>	TTGCTGCCAGACTTCGATGA	20	56.7	126
<i>NAC68_R</i>	GAACCACTCATCTTCCTTCTC	21	52.6	
<i>CAT_F</i>	CCCCATTCCAAATCGTATCG	20	54.6	165
<i>CAT_R</i>	CTCCGGGTTGCTTGAAATCG	20	57.8	

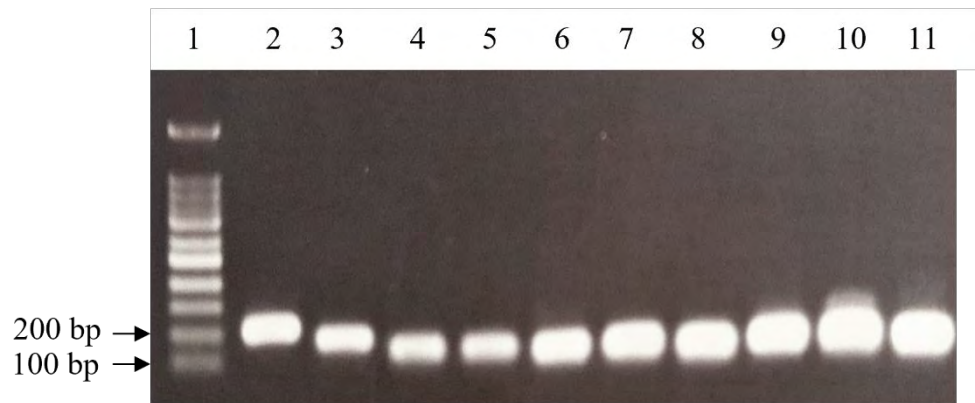


Figure 4.7: The AGE results showing the successful amplification of all targeted amplicons. The amplified amplicons corresponded to 10 genes including *RPS2* (Lane 2), *CAT* (Lane 3), *NAC68* (Lane 4), *Cytochrome P450* (Lane 5), *ChiH* (Lane 6), *PR1* (Lane 7), *WRKY50* (Lane 8), *PAL* (Lane 9), *WRKY22* (Lane 10) and *CEBiP* (Lane 11) fragments. Amplicon size was estimated using 100 bp DNA ladder (Lane 1).

4.6.1 Sequence analysis

To confirm the identity and specificity of the amplified fragments, PCR products were sequenced. The chromatogram obtained for both forward and reverse primer pair sequences for all the target genes are shown in Appendix C and the sequencing data were subjected to BLAST analysis using two databases: Phytozome (Figure 4.8) and NCBI (Figure 4.9). The similarity between the query sequences and the target genes' sequences in the GenBank was 98.46 %, 99.10 %, 100 %, 100 %, 96.97 %, 100 %, 98.33 %, 97.78 % and 97.30 % for *PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*, *Cytochrome P450*, *NAC68* and *CAT*, respectively indicating their specificity.



(a) *PR1*

Figure 4.8: The BLAST result from Phytozome database for *PR1* (a), *WRKY22* (b), *WRKY50* (c), *PAL* (d), *CEBiP* (e), *ChiH* (f), *Cytochrome P450* (g), *NAC68* (h) and *CAT* (i) genes.



(b) *WRKY22*

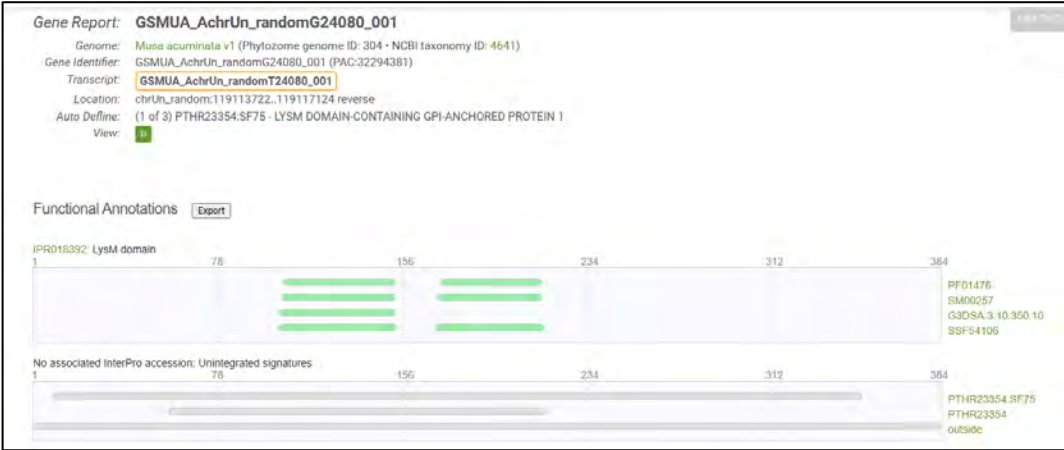


(c) *WRKY50*



(d) *PAL*

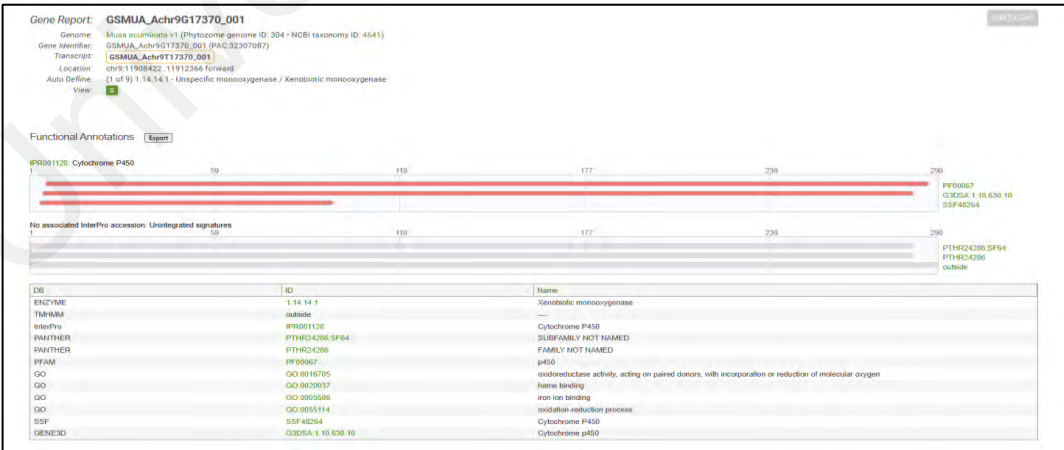
Figure 4.8, continued.



(e) *CEBiP*



(f) *ChiH*



(g) *Cytochrome P450*

Figure 4.8, continued.



(h) *NAC68*



(i) *CAT*

Figure 4.8, continued.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis pathogenesis-related protein 1C-like (LOC103989043)	Musa acuminata subsp. malaccensis	113	113	98%	2e-21	98.46%	692	XM_009390669.2
☒	Musa acuminata subsp. malaccensis strain Doubled-haploid Pahang (DH-Pahang) genome assembly	Musa acuminata subsp. malaccensis	113	388	98%	2e-21	98.46%	34728925	HG996467.1

(a) *PR1*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis probable WRKY transcription factor 21 (LOC103989043)	Musa acuminata subsp. malaccensis	198	198	99%	1e-46	99.10%	1811	XM_009418844.2
☒	PREDICTED: Musa acuminata subsp. malaccensis probable WRKY transcription factor 21 (LOC103989043)	Musa acuminata subsp. malaccensis	198	198	99%	1e-46	99.10%	2043	XM_009418843.2

(b) *WRKY22*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis probable WRKY transcription factor 50 (LOC103989043)	Musa acuminata subsp. malaccensis	1125	1125	100%	0.0	100.00%	743	XM_009410651.2
☒	PREDICTED: Musa acuminata subsp. malaccensis probable WRKY transcription factor 50 (LOC103989043)	Musa acuminata subsp. malaccensis	1066	1066	100%	0.0	98.52%	735	XM_009410652.2

(c) *WRKY50*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis phenylalanine ammonia-lyase-like (LOC103989043)	Musa acuminata subsp. malaccensis	215	215	93%	1e-51	100.00%	2563	XM_009419417.2
☒	Musa acuminata AAA Group cultivar Cavendish phenylalanine ammonia-lyase (PAL1) mRNA, partial cds	Musa acuminata subsp. malaccensis	215	215	93%	1e-51	100.00%	1818	KF582545.1

(d) *PAL*

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis lysM domain-containing GPI-anchored protein	Musa acuminata subsp. malaccensis	110	110	100%	2e-20	96.97%	1651	XM_009389909.2
☒	Musa acuminata subsp. malaccensis strain Doubled-haploid Pahang (DH-Pahang) genome assembly	Musa acuminata subsp. malaccensis	110	110	100%	2e-20	96.97%	47435180	HG996474.1

(e) *CEBiP*

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☐	PREDICTED: Musa acuminata subsp. malaccensis magnesium-chelatase subunit ChiH, c...	Musa acuminata subsp. malaccensis	wild Mal...	214687	233	233	96%	4e-57	100.00%	4615	XM_009391351.2
☐	Musa troglodytarum cultivar karat chromosome 2	Musa troglodytarum	NA	320322	233	233	96%	4e-57	100.00%	56266567	CP097504.1

(f) *ChiH*

Figure 4.9: The BLAST result from NCBI database showing the top BLAST hits with percentage similarity of > 95 % for *PR1* (a), *WRKY22* (b), *WRKY50* (c), *PAL* (d), *CEBiP* (e), *ChiH* (f), *Cytochrome P450* (g), *NAC68* (h) and *CAT* (i) genes.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	PREDICTED: Musa acuminata subsp. malaccensis probable cytochrome P450 313a1 (LOC1...	Musa acumin...	211	211	100%	2e-50	98.33%	2163	XM_009419626.2
☒	Musa acuminata subsp. malaccensis strain Doubled-haploid Pahang (DH-Pahang) genome a...	Musa acumin...	211	417	100%	2e-50	98.33%	47435180	HG998474.1

(g) Cytochrome P450

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	Musa ABB Group cultivar Karibale Monthan NAC domain containing transcription factor NAC68 mRNA c...	Musa ABB Group	159	159	83%	6e-35	98.89%	897	KR078315.1
☒	PREDICTED: Musa acuminata subsp. malaccensis NAC domain containing protein 68 like (LOC103992...	Musa acuminat...	154	154	83%	3e-33	97.78%	1109	XM_009412203.2

(h) NAC68

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	Musa acuminata subsp. malaccensis strain Doubled-haploid Pahang (DH-Pahang) genome assembly ch...	Musa acuminat...	143	143	53%	1e-29	96.51%	37945898	HC996466.1
☒	Musa troglodytarum cultivar karai chromosome 1	Musa troglodyta...	137	137	53%	4e-28	95.35%	67423607	CP097502.1
☒	PREDICTED: Musa acuminata subsp. malaccensis catalase isozyme A (LOC103983085) mRNA	Musa acuminat...	63.9	63.9	22%	8e-06	97.30%	1854	XM_009400246.2

(i) CAT

Figure 4.9, continued.

4.7 RNA extraction, DNase treatment and cDNA synthesis

Total RNA samples were successfully extracted from all four groups at four different timepoints in triplicates as mentioned in section 3.12. Clear bands of both 28S and 18S rRNA observed in AGE analysis indicated the success of RNA extraction. To remove genomic DNA, DNase treatment was performed on all samples and successful DNase treatment was confirmed by the absence of high molecular weight fragments in the gel (Appendix D). The A260/A280 ratios of the RNA extracts were in the range of 1.8 - 2.0 (Appendix E). Figure 4.10 shows the AGE image for RNA extracts before (Figure 4.10a) and after DNase treatment (Figure 4.10b) of representative samples. Subsequently, RNA samples that have been treated with DNase were then converted into cDNA. Successful cDNA synthesis was verified by PCR using housekeeping gene, *RPS2* indicated by the bands present at ~200 bp (Figure 4.11).

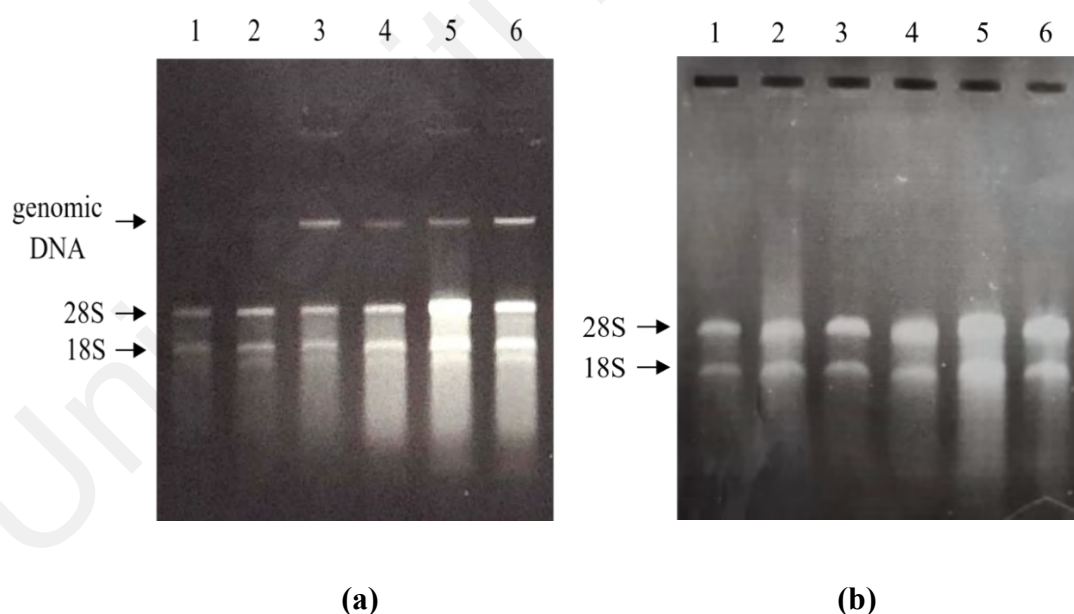
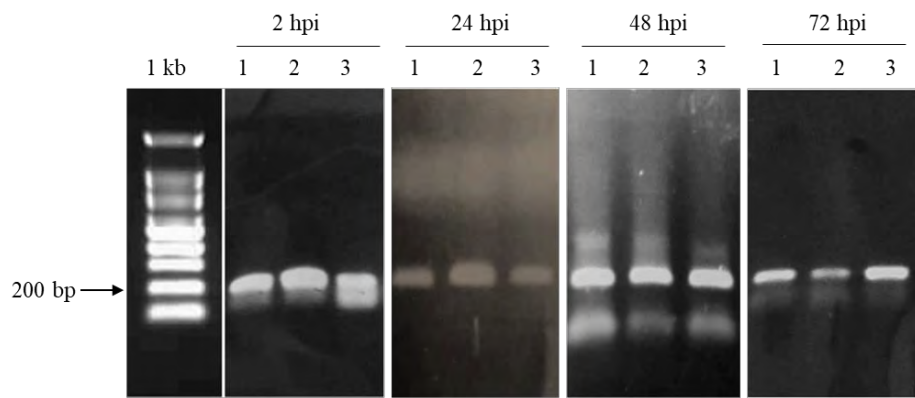
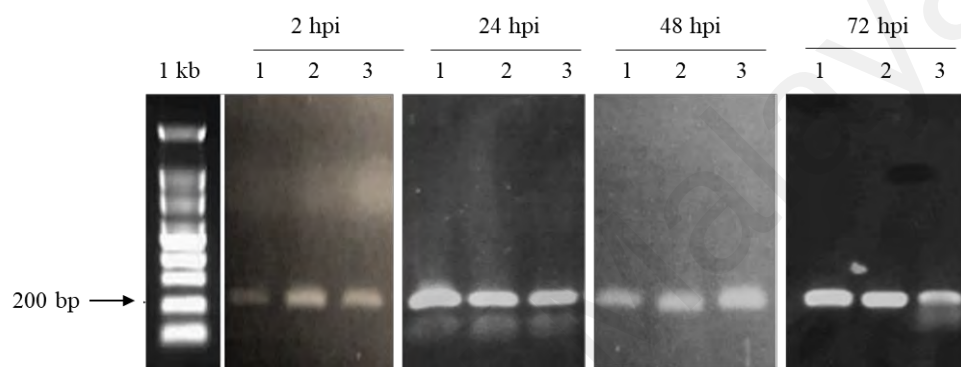


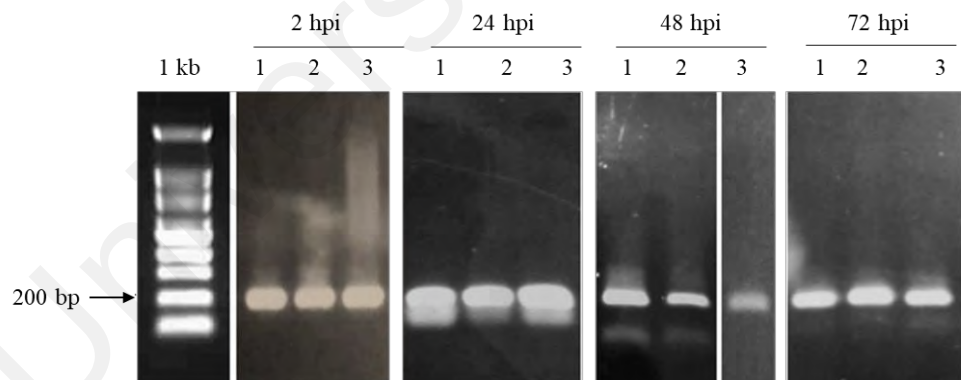
Figure 4.10: RNA extraction before (a) and after (b) DNase treatment. The AGE shows representative samples from treated (plasma) + *Foc* inoculated (Group 1) (lane 1, 2 and 3) and untreated (non-plasma) + *Foc* inoculated (Group 2) (lane 4, 5, and 6) at 2 hpi.



(a) Treated (plasma) + *Foc* inoculated (Group 1)

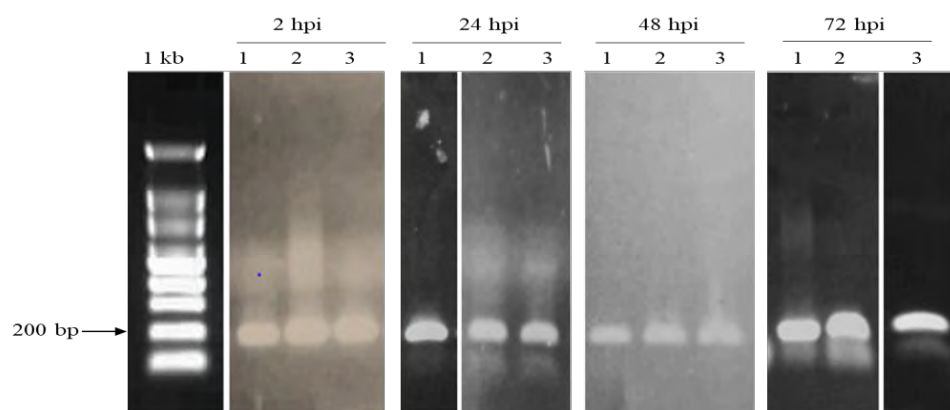


(b) Untreated (non-plasma) + *Foc* inoculated (Group 2)



(c) Treated (plasma) + non-*Foc* inoculated (Group 3)

Figure 4.11: Validation of successful cDNA synthesis. Gel image shows amplification of *RPS2* fragments from treated (plasma) + *Foc* inoculated (Group 1) (a), untreated (non-plasma) + *Foc* inoculated (Group 2) (b), treated (plasma) + non-*Foc* inoculated (Group 3) (c) and untreated (non-plasma) + non-*Foc* inoculated (Group 4) (d) samples. 1 kb indicates the ladder used; 1-3' indicates cDNA samples in triplicates for each timepoint of each group. Bands with estimated size of 200 bp verified the successful cDNA conversion.



(d) Untreated (non-plasma) + non-*Foc* inoculated (Group 4)

Figure 4.11, continued.

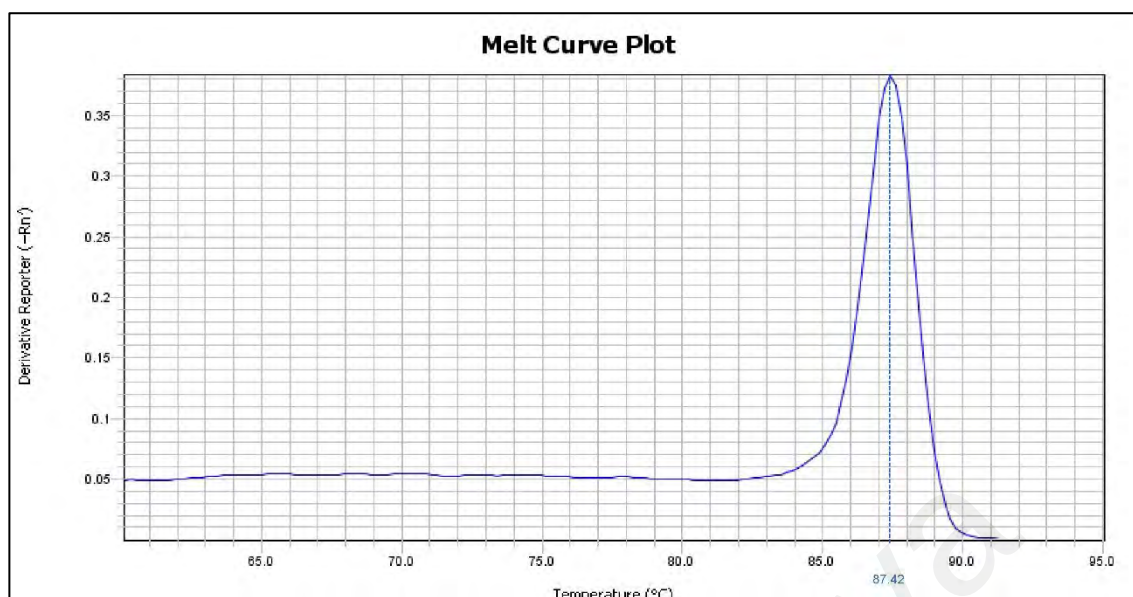
4.8 Quantitative real-time polymerase chain reaction (RT-qPCR) analysis

4.8.1 Primer efficiency test

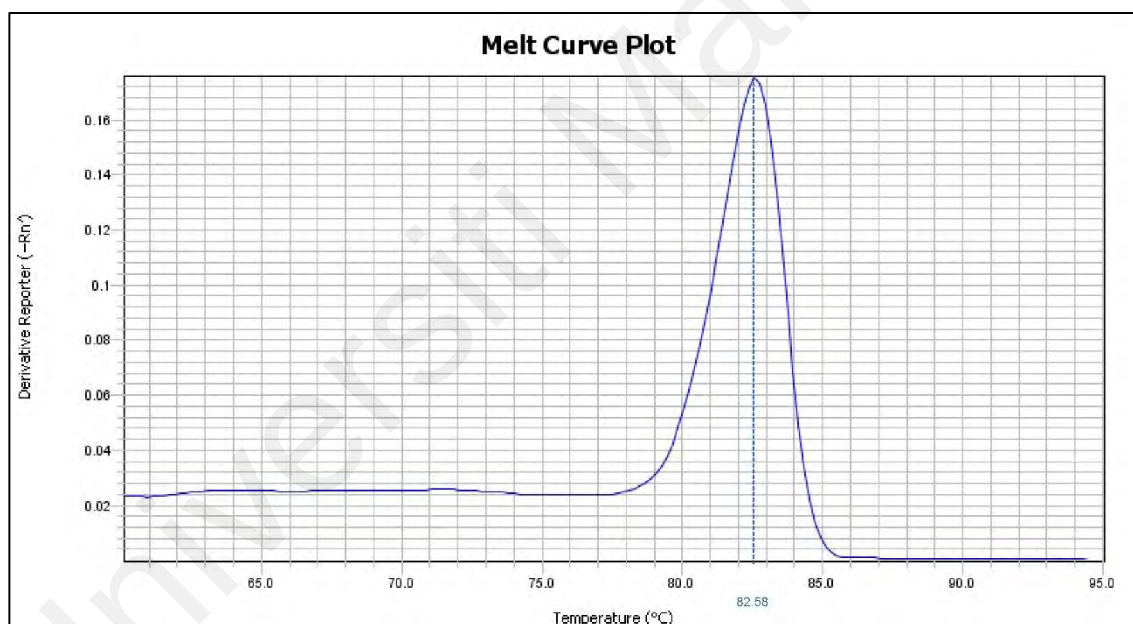
To ensure the efficiency of the primers used in this study, melt curve analysis was carried out and PCR primer efficiencies were calculated. Figure 4.12 shows melt curves of *PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*, *Cytochrome P450*, *NAC68*, *CAT* and *RPS2* (housekeeping) genes. Single peak was observed for all the target and housekeeping genes indicating their specificity. The PCR primer efficiency of all the primer pairs used in this study ranged between the 90 % – 110 %. This indicates that the primer efficiencies are comparable for all the genes of interest and housekeeping gene used in this study (Table 4.4).

Table 4.4: PCR primer efficiencies for genes used in this study.

Primer pair	Efficiency (%)
<i>PR1</i>	94.15
<i>WRKY22</i>	96.16
<i>WRKY50</i>	108.75
<i>PAL</i>	99.05
<i>CEBiP</i>	92.83
<i>ChiH</i>	96.39
<i>Cytochrome P450</i>	92.40
<i>NAC68</i>	95.86
<i>CAT</i>	103.19
<i>RPS2</i>	107.95

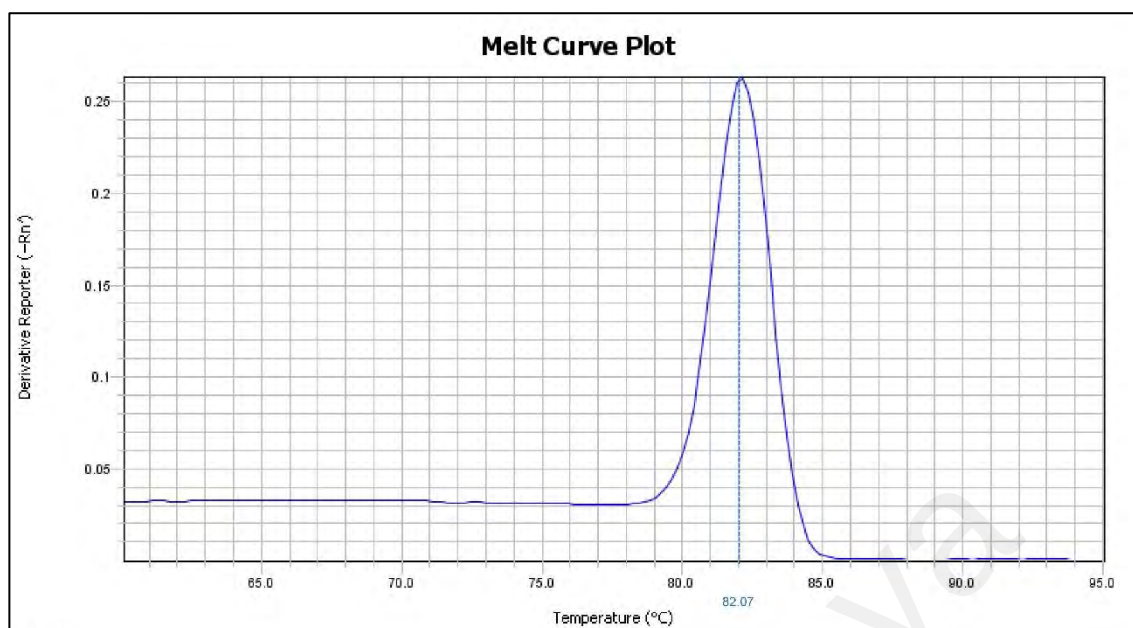


(a) *PR1*

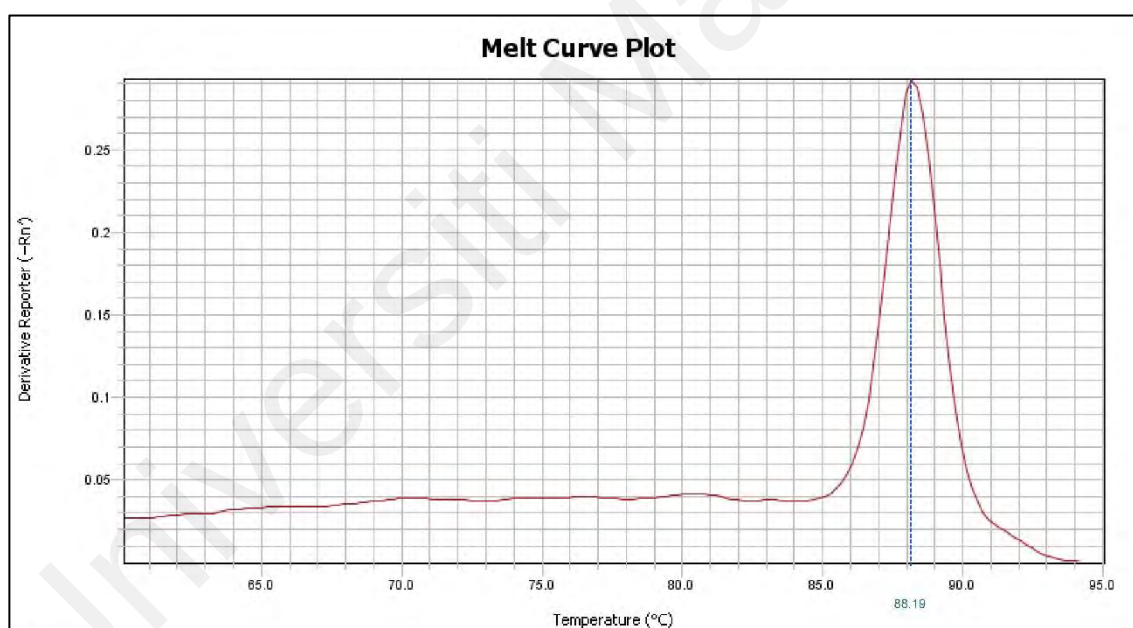


(b) *WRKY22*

Figure 4.12: Melt curves of *PR1* (a), *WRKY22* (b), *WRKY50* (c), *PAL* (d), *CEBiP* (e), *ChiH* (f), *Cytochrome P450* (g), *NAC68* (h), *CAT* (i), and *RPS2* (j) genes show single peak with T_m value (°C) of 87.42, 82.58, 82.07, 88.19, 80.43, 89.46, 79.36, 81.38, 81.39 and 81.1, respectively.

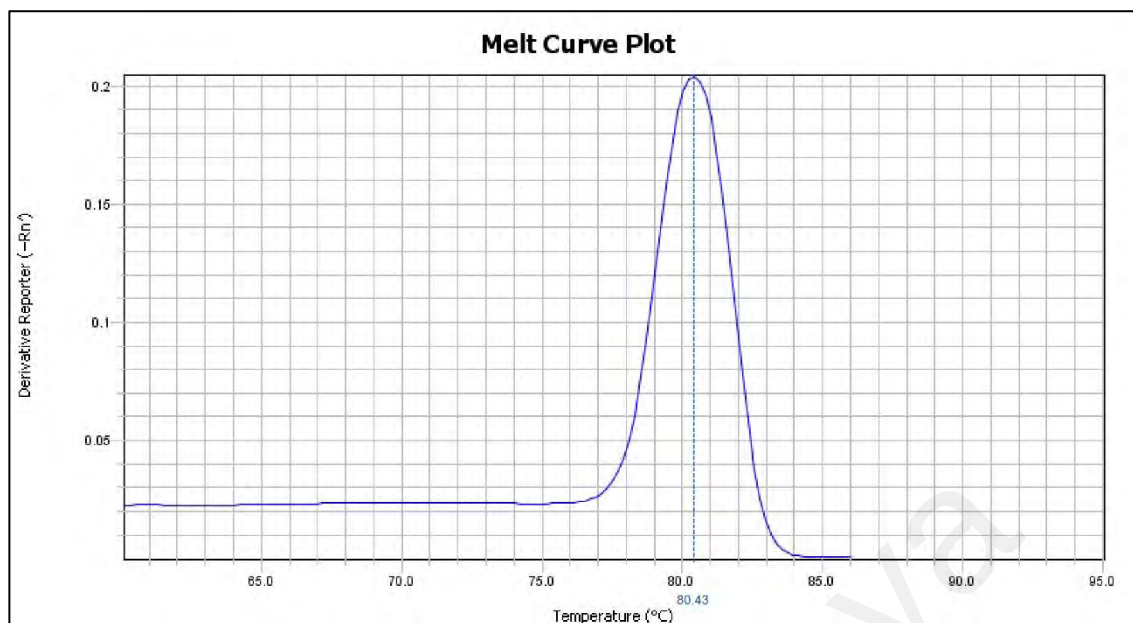


(c) *WRKY50*

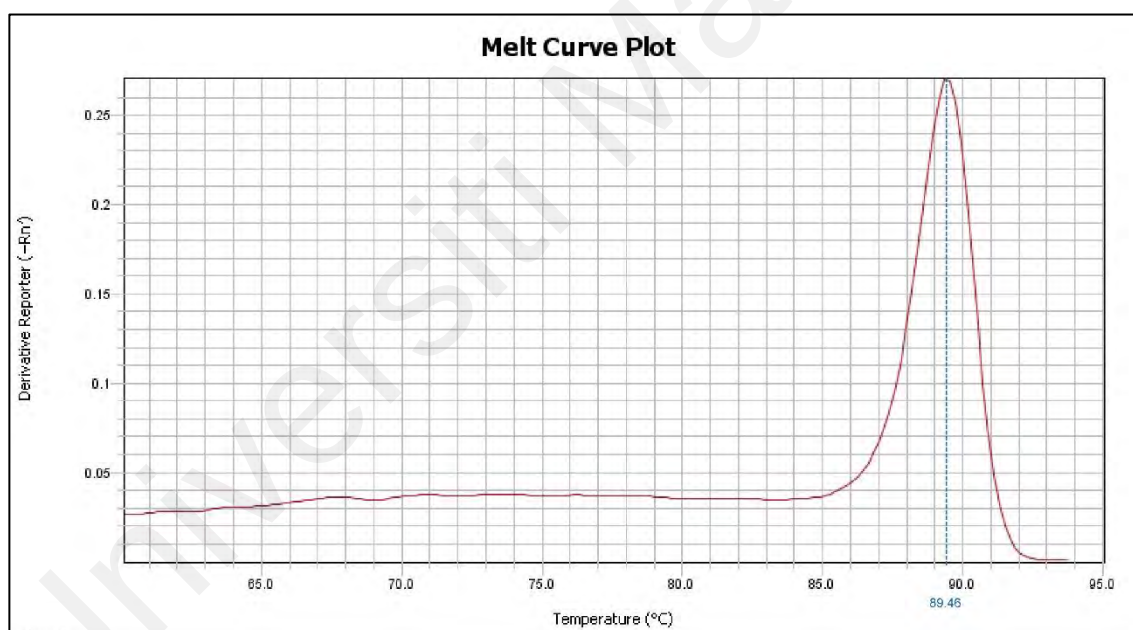


(d) *PAL*

Figure 4.12, continued

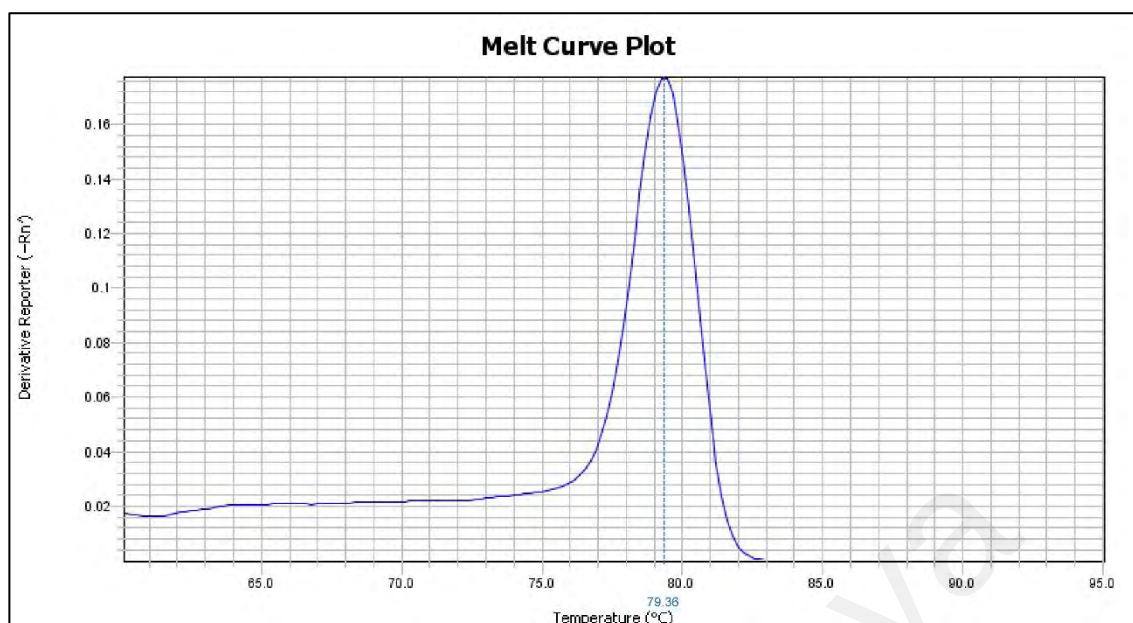


(e) *CEBiP*

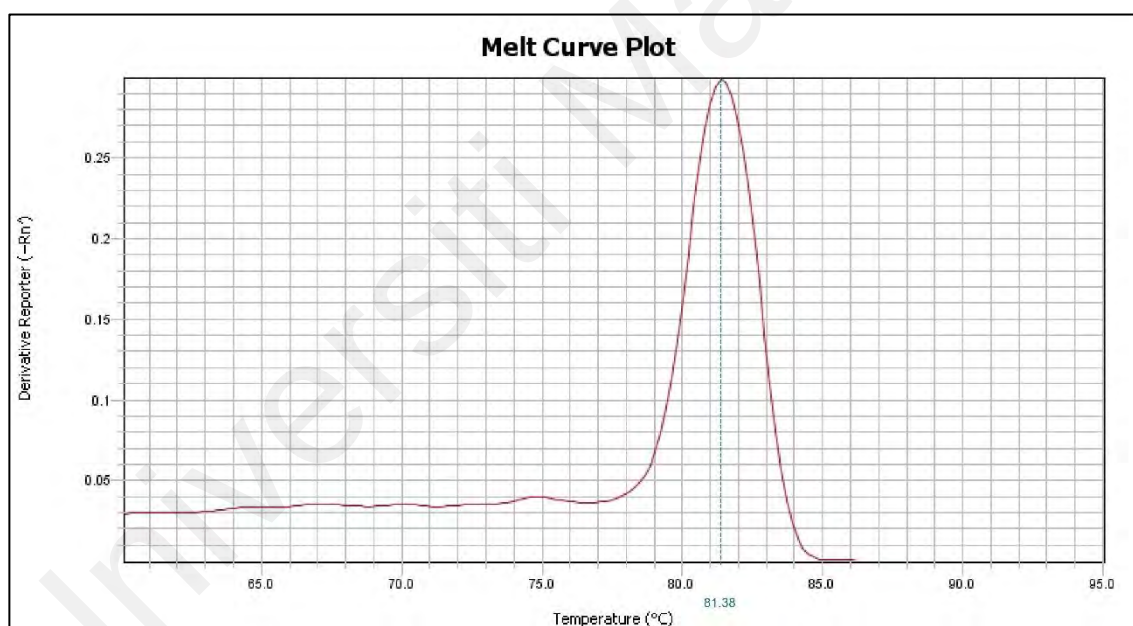


(f) *ChiH*

Figure 4.12, continued.

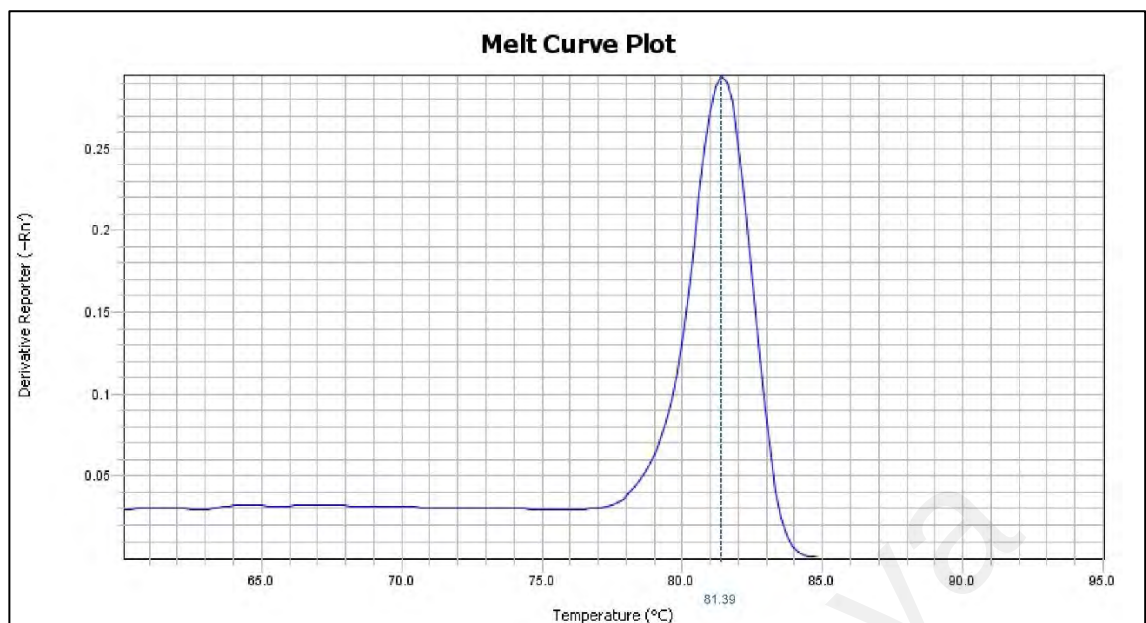


(g) *Cytochrome P450*

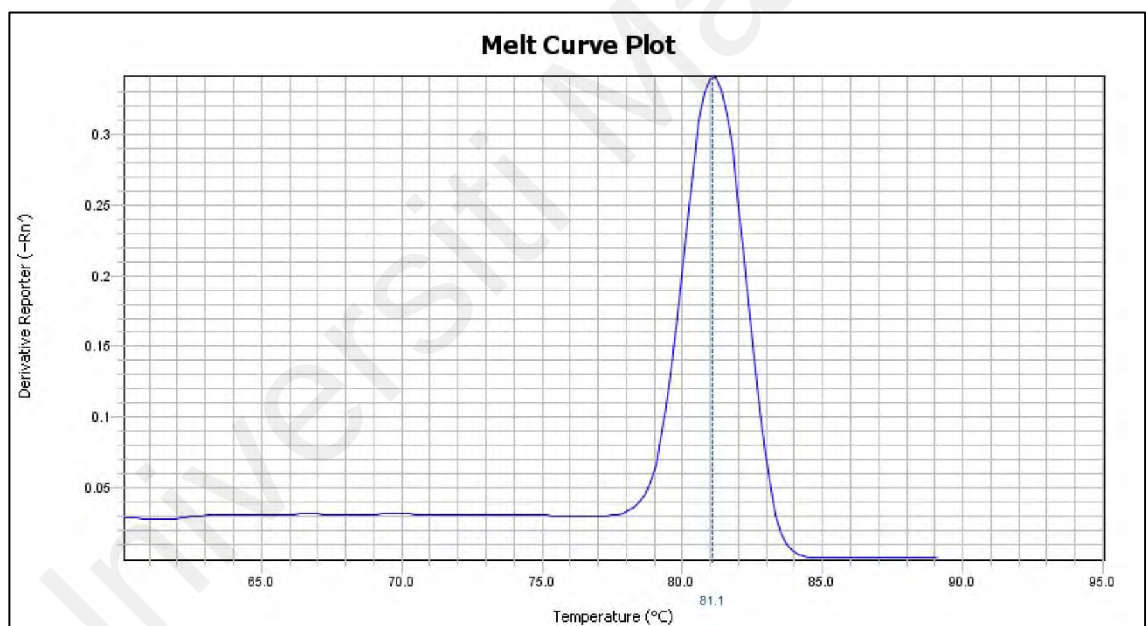


(h) *NAC68*

Figure 4.12, continued.



(i) *CAT*



(j) *RPS2* (housekeeping gene)

Figure 4.12, continued.

4.8.2 Gene expression analysis of defence and growth-related genes

In response to plasma treatment and *Foc* TR4 infection, the gene expression dynamics of both defence-related and growth-related genes were examined. The selected genes displayed distinct patterns of expression within three days of *Foc* TR4 infection when normalized against *RPS2* gene (Appendix F). Figure 4.13 shows the relative expression profiles of nine genes - *PRI*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*, *Cytochrome P450*, *NAC68* and *CAT* genes at different time points (2, 24, 48, and 72 hours post *Foc* TR4 inoculation) across the three groups, treated (plasma) + *Foc* inoculated (Group 1), untreated (non-plasma) + *Foc* inoculated (Group 2) and treated (plasma) + non-*Foc* inoculated (Group 3).

The expression of *PRI* gene was found to be upregulated and showed higher expression in *Foc* inoculated plants (Group 1 & 2) compared to non-inoculated plants (Group 3) starting from 2 hpi. Notably, significant increase (63-fold change) in *PRI* expression was observed in plasma-treated plants (Group 1) at 72 hpi, distinguishing it from the other treatment groups (Figure 4.13a). Similarly, *WRKY22* gene displayed increased expression in *Foc* inoculated plants (Group 1 & 2) compared to non-inoculated plants (Group 3) starting from 24 hpi. However, significant increase (18-fold change) of *WRKY22* expression was observed in plasma-treated plants (Group 1) at 72 hpi compared to the other treatment group plants (Figure 4.13b).

Next, *WRKY50* gene demonstrated increased expression in *Foc* inoculated plants (Group 1 & 2) compared to non-inoculated plants (Group 3) starting from 48 hpi. The expression peaked at 72 hpi (4-fold change) in *Foc* inoculated plants that was untreated (Group 2) compared to plasma-treated (Group 1) plants. However, the change was not statistically significant between these two groups at 72 hpi. As for non-*Foc* inoculated plants (Group 3), no significant change was observed from 2 hpi with only upregulation observed at 24 hpi (Figure 4.13c).

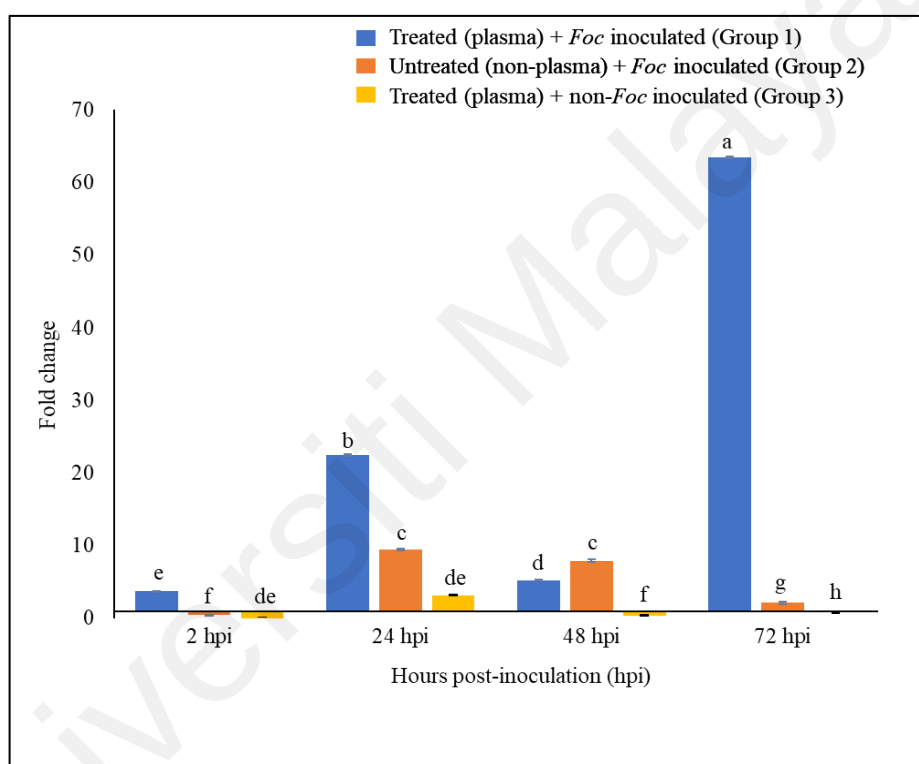
The *PAL* gene exhibited an increase in expression from 24 hpi until peaking at 48 hpi in untreated (non-plasma) + *Foc* inoculated plants (Group 2), while treated (plasma) + *Foc*-inoculated plants (Group 1) displayed the highest *PAL* expression at 24 hpi before subsequent decline. In non-*Foc* inoculated plants (Group 3), the highest expression was observed at 24 hpi followed by a decrease (Figure 4.13d).

CEBiP gene expression in non-*Foc* inoculated plants (Group 3) remained unchanged at all timepoints. In the *Foc* inoculated plants, *CEBiP* gene exhibited upregulation at 2 hpi and at 72 hpi in plasma-treated (Group 1) and untreated (Group 2) groups respectively. The highest expression was at 72 hpi (4-fold change) in group 2. However, change observed between this two upregulation is not statistically significant (Figure 4.13e). For *ChiH* gene, a significant upregulation (35-fold change) was only observed only in untreated (non-plasma) + *Foc* inoculated plants (Group 2) at 72 hpi (Figure 4.13f).

The *Cytochrome P450* gene showed consistent upregulation with higher expression in treated (plasma) + *Foc* inoculated plants (Group 1) at all time points compared to the other treatment groups. The peak (5-fold change) occurred in group 1 at 48 hpi. Conversely, untreated (non-plasma) + *Foc* inoculated plants (Group 2) exhibited upregulation but with lower expression from 2 hpi, gradually decreasing afterward. Non-*Foc* inoculated plants (Group 3) displayed upregulated expression at 24 hpi followed by a decrease (Figure 4.13g).

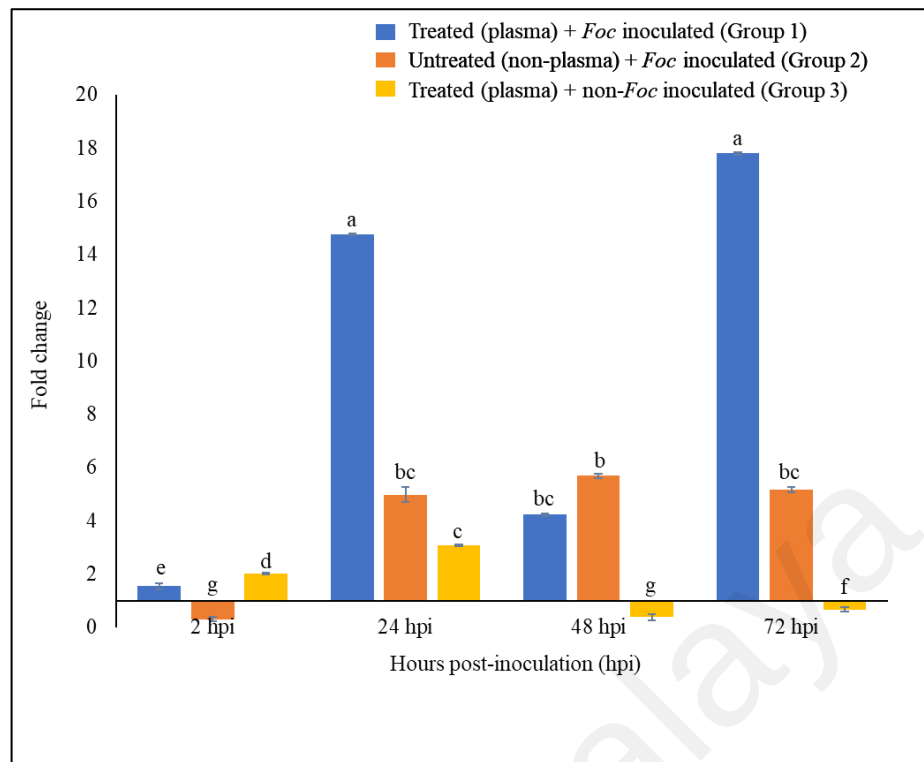
NAC68 gene expression significantly peaked at 72 hpi (7-fold change) in treated (plasma) + *Foc* inoculated plants (Group 1). For the non-*Foc* inoculated plants (Group 3), upregulation observed at 2 hpi where it downregulated afterwards. For untreated (non-plasma) + *Foc* inoculated plants (Group 2), upregulation observed from 24 hpi. However, the expression decreased afterwards (Figure 4.13h).

The expression levels of *CAT* gene steadily increased over time in *Foc* inoculated plants (Group 1 and 2) from 2 hpi. However, the expression downregulated in the untreated plants (non-plasma) (Group 2) at 72 hpi. Highest expression (29-fold change) of *CAT* gene was observed in plasma-treated plants (Group 1) at 72 hpi. *CAT* gene also showed steady increase with lower expression in non-*Foc* inoculated plants (Group 3) from 2 hpi but downregulated at 72 hpi (Figure 4.13i).

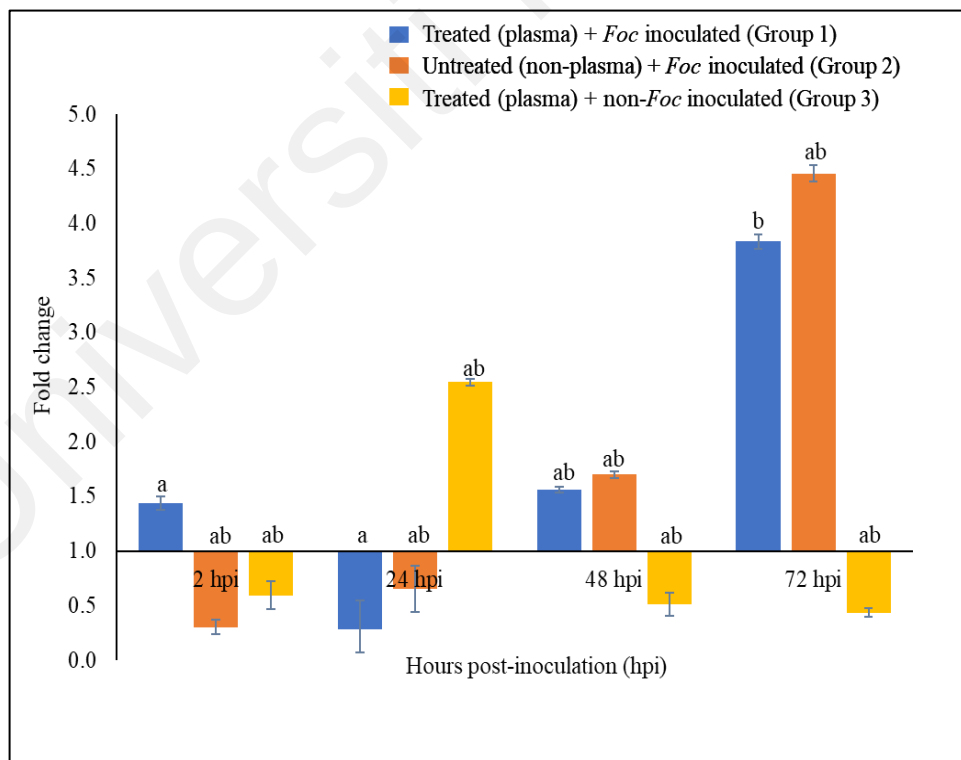


(a) *PR1*

Figure 4.13: Relative expression value of *PR1* (a), *WRKY22* (b), *WRKY50* (c), *PAL* (d), *CEBiP* (e), *ChiH* (f), *Cytochrome P450* (g), *NAC68* (h) and *CAT* (i) genes by qPCR in three different groups; treated (plasma) + *Foc* inoculated (Group 1), untreated (non-plasma) + *Foc* inoculated (Group 2) and treated (plasma) + non-*Foc* inoculated (Group 3). Target gene expression level was normalized to RPS2 reference gene. Relative expression value of the target genes was calculated based on the fold change value of ‘untreated (non-plasma) + non-*Foc* inoculated’ (Group 4) plants at 2 hpi. The bars represent the fold changes of the expression levels. All data were subjected to two-way ANOVA. The data shown are expressed as the mean (%) of three technical replicates from the pool of three biological replicates $\pm$ standard error. Error bar represents standard error; same letter denotes not significant ($p > 0.05$) by Tukey’s test.

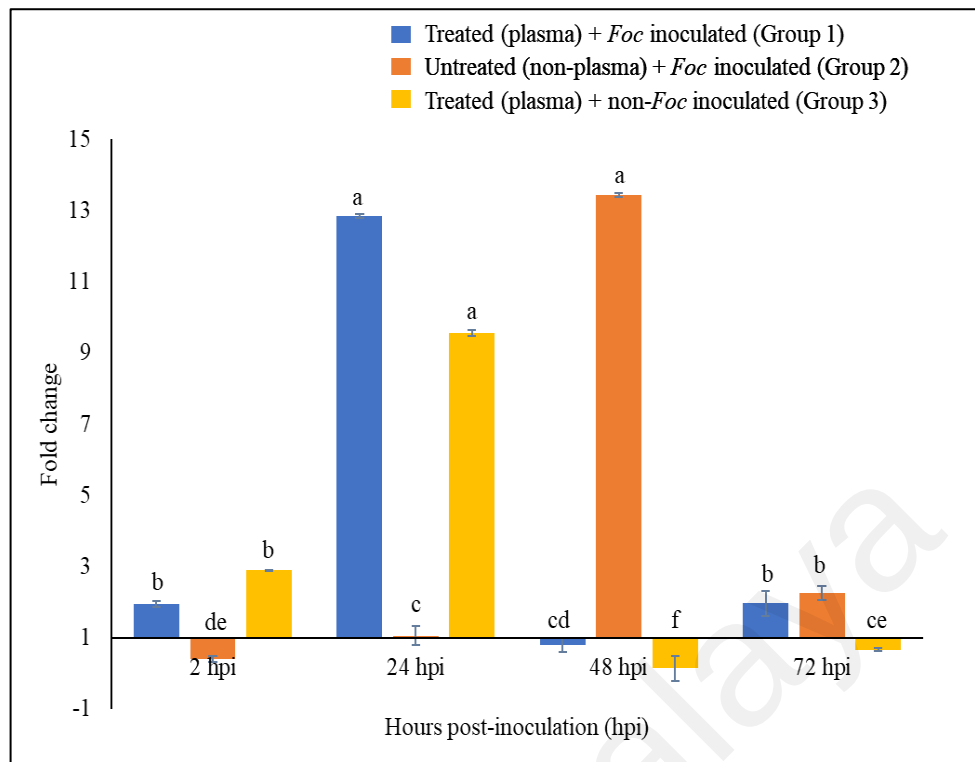


(b) *WRKY22*

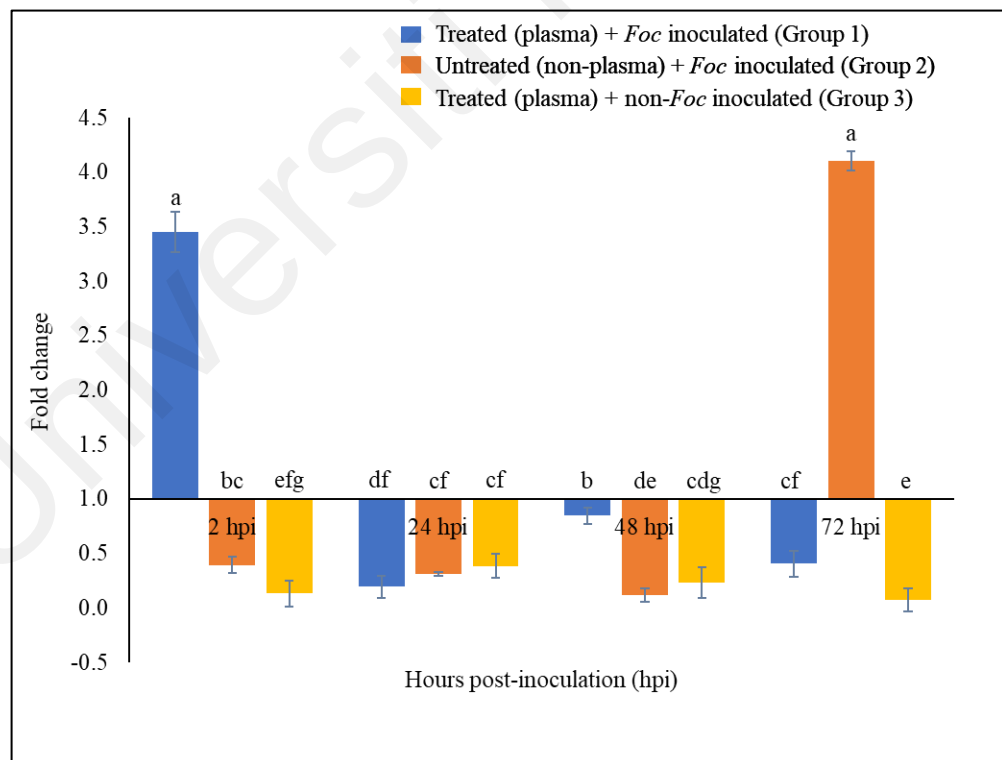


(c) *WRKY50*

Figure 4.13, continued.

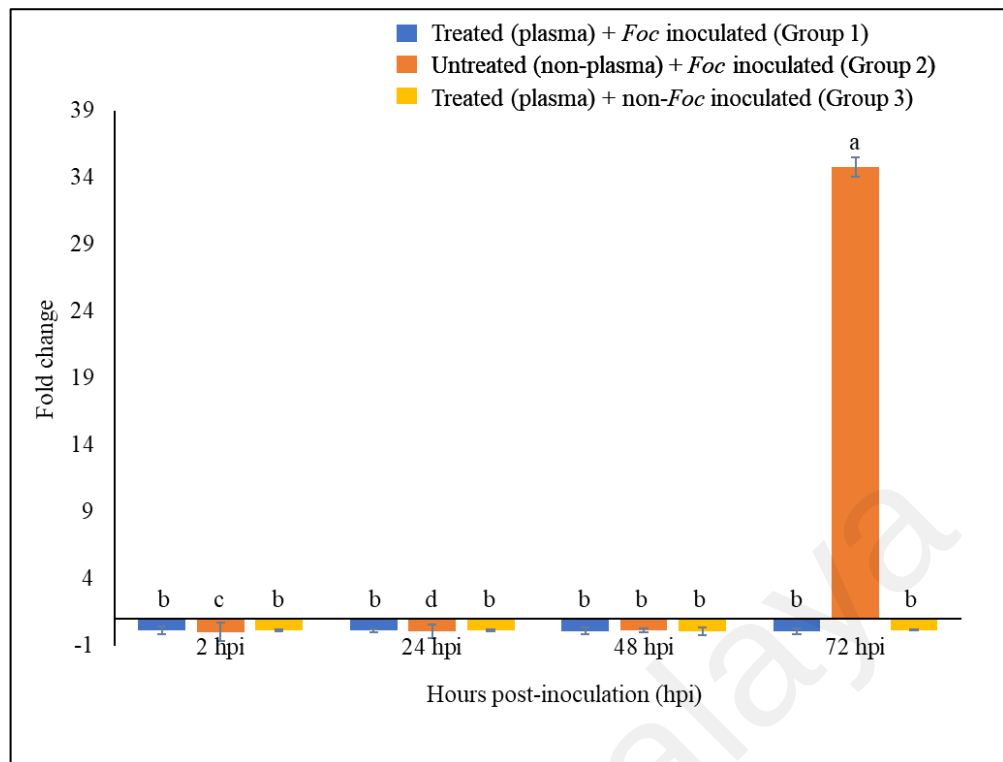


(d) *PAL*

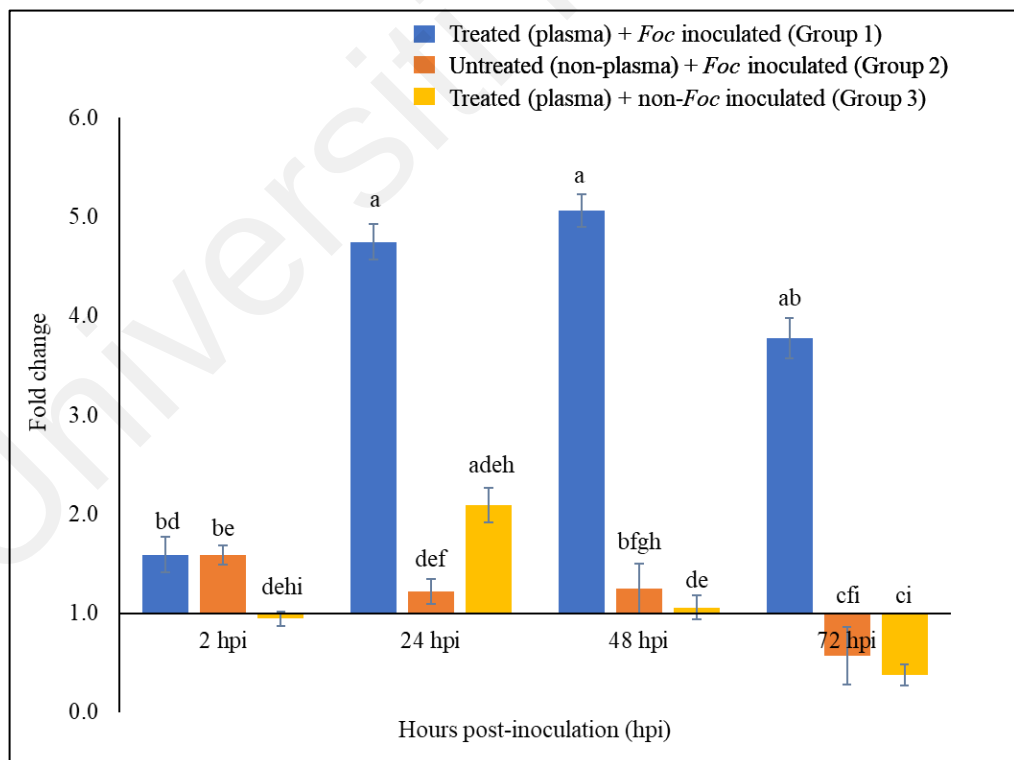


(e) *CEBiP*

Figure 4.13, continued.

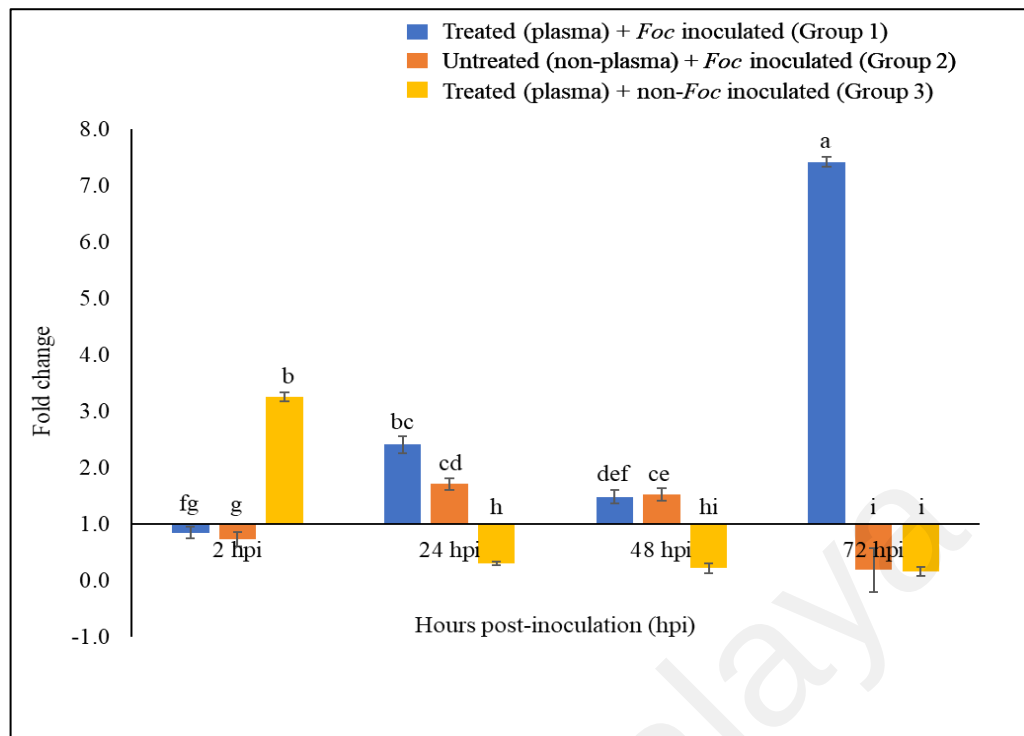


(f) *ChiH*

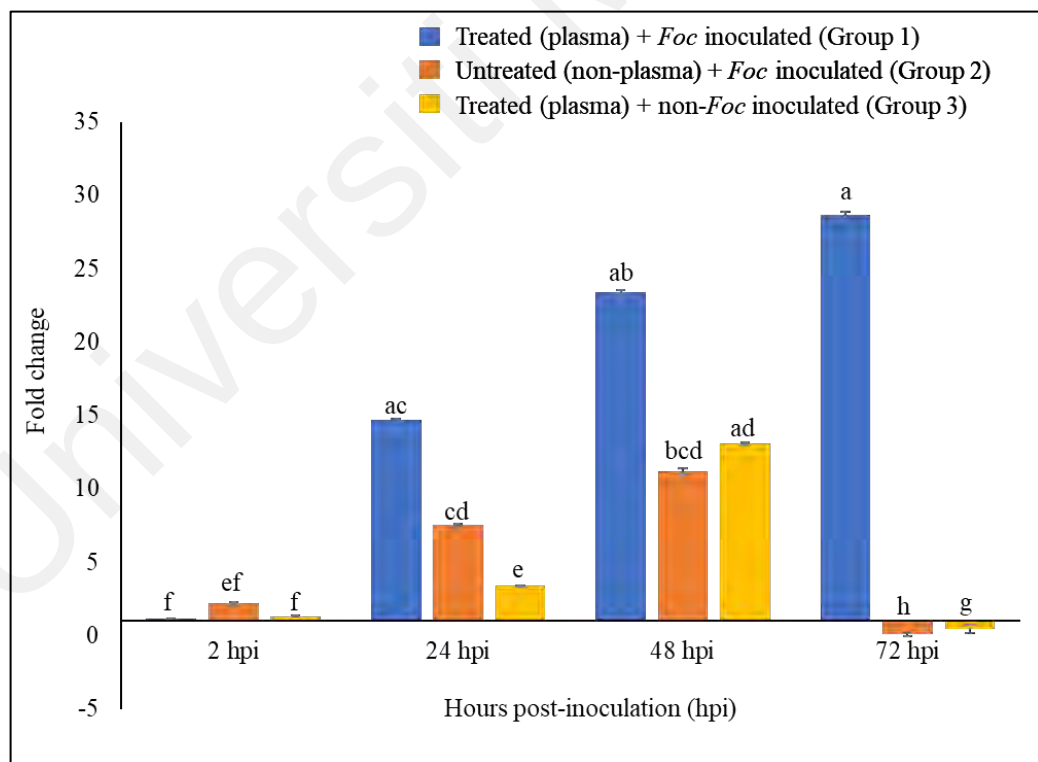


(g) *Cytochrome P450*

Figure 4.13, continued.



(h) *NAC68*



(i) *CAT*

Figure 4.13, continued.

CHAPTER 5: DISCUSSION

Control and management of *Foc* TR4 remains a main challenge in ensuring high banana production in the country. Multiple research projects collectively exploring various approaches, including chemical controls, biological controls, modification of cultural practices and resistance breeding to reduce the severity of Fusarium wilt. However, since *Foc* TR4 can survive in the absence of host, managing this disease via agronomic practices such as crop rotation is inefficient. Moreover, sustainable control of the disease has not been achieved by means of chemical management measures such as corm injection and soil drenching with carbendazim fungicide, as their repeated application raises environmental concerns (Damodaran *et al.*, 2020).

Researchers have shifted their attention towards the selection of resistant varieties to overcome *Foc* TR4 (Cheng *et al.*, 2019). Studies have reported some advances using resistance breeding for developing resistant varieties and hybrids against *Foc* race 1, but to date no successful reports available on the development of resistant varieties against *Foc* TR4 (Rebouças *et al.*, 2018; Ribeiro *et al.*, 2018; Gonçalves *et al.*, 2019). Cultivation of tolerant or resistant varieties remains to be an effective control strategy to overcome the disease and loss caused by Fusarium wilt (Baharum *et al.*, 2018). However, the existence of poor fertility nature in bananas is limiting the improvement programs by conventional breeding. Besides, producing resistant varieties are commonly scarce, commercially unacceptable and unproductive (Zorrilla-Fontanesi *et al.*, 2020).

From all these limitations arise, the important question of how much yield loss can be avoided by the prevention of *Foc* TR4 entry or by delaying their spread must be given priority (Staver *et al.*, 2020). Thus, this study has highlighted the need for a modern eco-agricultural approach that is economically viable and environmentally friendly which is the CP treatment to produce improved banana plants that are more tolerant against *Foc* TR4. CP treatment plays crucial role in crop optimization by plant growth enhancement,

rejuvenation and yield maximization. However, the effect of CP treatment on plant disease control is not well studied especially on vegetatively propagated crops such as banana.

5.1 Cold plasma (CP) treatment

In this current study, we investigate the effect of CP treatment on the growth and Fusarium wilt severity in *M. acuminata* cv. 'Berangan'. In addition, we attempted to elucidate the mechanism of action and effect of CP treatment on the Fusarium wilt disease control through gene (growth and defence) expression study. In the present study, the parameters of CP treatment were chosen based on literatures (Sandanuwan *et al.*, 2020). Treating Cavendish bananas with CP for 30 secs with voltage of 15 kV was found to positively influence the rate of post-harvest crown rot disinfection in bananas thus being able to suppress the natural infection (Sandanuwan *et al.*, 2020). However, in the present study banana plantlets were treated with CP for lower duration (15 seconds) with voltage of 15 kV. These mild parameters were selected due to the nature of soft tissues of the tissue culture derived plantlets used in this study. Moreover, longer exposure with high voltage of CP was reported to damage the banana peel and cause chlorophyll degradation which affected the fruit colouring (Yagual *et al.*, 2023). Besides, high power plasma also found to reduce the germination rate in alfalfa plant (Jinkui *et al.*, 2018). Based on the present study, the 'Berangan' tissue culture plantlets treated with plasma showed no visible damage following CP treatment. The plants were also healthy throughout the study indicating that the chosen parameters are ideal and not detrimental to the banana plant tissues.

5.2 Fusarium wilt bioassay

In the context of the *Fusarium* bioassay, the spore concentration of *Foc* TR4 was standardized at 10^6 spores/mL. This agrees with many other studies carried out as it was found to be optimal for consistent *Fusarium* wilt symptom progression (Guo *et al.*, 2014; Pérez Vicente *et al.*, 2014; Mohd-Yusuf *et al.*, 2019). This is because lower concentrations have reported to not being able to produce or produce inconsistent reproducible phenotypic symptoms as stated by the DSI values (Mak *et al.*, 2004; Mohd-Yusuf *et al.*, 2019). Spore concentrations used in challenge experiments must be consistent to make sure similar surface area to volume ratio for inoculum contact. This is because plant responses and gene expression profiles will be affected by any variations during challenge experiments (Pérez Vicente *et al.*, 2014).

The simple and reliable root dipping technique was selected for the *Foc* TR4 challenge experiment as it mimics the natural mode of infection as *Foc* infects through root of banana plants. Studies have reported the success of using root dipping method such as in strawberry and pepper plants (Masheva and Todorova, 2013). ‘Berangan’ plants which are two months old were selected for the infection study as they are easier to handle and root damage during the procedure can be minimized (Mohd-Yusuf *et al.*, 2019). As for the duration of inoculation which is another important factor, two-hours of inoculation was reported to be effective in producing expected disease progression in ‘Berangan’ cultivars which are susceptible to *Foc* TR4 infection (Mohd-Yusuf *et al.*, 2019). Double tray method was used during the challenge experiment as it provides safe procedure (Mak *et al.*, 2004). This method provides a controlled environment to observe plant response against pathogen attack and requires only minimal space. Besides, pathogen contaminated water will be drained into the bottom tray and thus avoiding contamination of other planting materials (Mohd-Yusuf *et al.*, 2019). Then, for the root sample collections, early infection hours were selected which are 2, 24, 48 and 72 hpi to study the expression profile

of early *Foc* TR4-responsive genes which are crucial to minimize the severity of Fusarium wilt infection.

5.3 The effect of plasma treatment on the growth of *M. acuminata* cv. 'Berangan'

Improving banana yield is the main concern of farmers and growth directly influences the yield. Thus, the first part of this study analysed the effect of CP treatment on the banana plant growth based on three parameters (height, number of leaf and stem diameter) which are the indicators of plant growth. As mentioned earlier in section 4.3, even though favourable growth improvements were observed, statistical analysis revealed that the observed improvement in plasma-treated plants are not significant. However, in general the plasma-treated plants showed better growth performances compared to untreated plants which suggest that the CP impact on growth improvement could be positive. Moreover, many studies have reported on the positive influence of CP on the growth parameters of tomato (Jiang *et al.*, 2014; Adhikari *et al.*, 2020), wheat (Jiafeng *et al.*, 2014), oilseed rape (Ling *et al.*, 2018; Mildažienė *et al.*, 2019), peanuts (Li *et al.*, 2016), sprouts (Xiang *et al.*, 2019), alfalfa seed (Jinkui *et al.*, 2018), sunflower (Tamosiune *et al.*, 2020) and grapes (Mujahid *et al.*, 2020).

One of the important factors that affects plant growth are nutrients. Based on Jiang *et al.* (2014), plasma treatment significantly increased calcium and boron uptake by plants compared to controls without plasma treatment which are essential for plant growth. Study on oilseed rape revealed that plasma treatment significantly improved their growth indicated by improved growth parameters such as plant height and stem diameter which is also in accordance with our study. The improvement in growth was supported by the improved wettability, permeability and water uptake of the seeds by CP treatment which are crucial for healthy plant growth (Ling *et al.*, 2018).

All these above studies provide an insight into the mechanisms involved in improving plant growth by CP treatment. Based on the findings from growth observation, it is important to note that plasma treatment did not cause any visible tissue damage and plants that have been treated with plasma can be acclimatized in a similar manner of plants that are untreated with plasma. Besides, it is also important to take into consideration that this is the pioneer study that uses plantlets as starting materials for CP treatment. Commonly, seeds were used in many studies on plasma effects on plants and significant increase was observed in the growth parameters tested (Jiang *et al.*, 2014; Ling *et al.*, 2015; Jinkui *et al.*, 2018; Adhikari *et al.*, 2020). Since this study used tissue culture plantlets, the influence of CP treatment might not be the same and a lot of optimizations need to be done to obtain a significant result. However, this study has shed some light on the potential use of plasma on the banana plantlets instead of the regular use of seeds which is not possible to be obtained from cultivated banana ('Berangan') as the cultivated banana are sterile.

5.4 The effect of plasma treatment on the Fusarium wilt disease progression in *M. acuminata* cv. 'Berangan'

The second part of this study focused on the effect of CP treatment on disease tolerance against *Foc* TR4 in the susceptible 'Berangan' cultivar. Studies have reported that the external and internal disease observation often do not associate with each other, and thus inconsistent results are produced. Infected plants might have no rhizome discoloration but with severe yellowing symptoms. This might be due to external factors such as lack of nutrient and unfavourable environment but not due to pathogen infection (Czembor *et al.*, 2015). Thus, to overcome this misinterpretation issue, this study correlates external and internal observations to produce a reliable disease scoring scale.

The data in section 4.4 and 4.5, reported both the external and internal disease observation respectively. Based on these results, it was demonstrated that CP treatment delayed the disease progression by one week (external observation) and slightly reduced

the Fusarium wilt disease severity (internal observation) although the treatment did not confer absolute resistance to *Foc* TR4 in 'Berangan' plants. This finding is similar to the previously reported study on tomato where plasma-treated plants showed slower disease progression with lesser disease severity compared to untreated plants (Jiang *et al.*, 2014).

There are several potential mechanisms through which plasma treatment could enhance tolerance or improve the response against pathogens. As highlighted earlier, the banana plants subjected to treatment exhibited no detrimental effects on growth; in fact, they displayed a general improvement in growth compared to the untreated counterparts. This enhanced health status observed in the treated plants could potentially impact their response to various stressors, including exposure to pathogens like *Foc*. Our findings suggest that the delayed disease progression and reduction observed in disease severity of 'Berangan' plants could be influenced by their improved healthy growing status observed in section 4.3. This result was in accordance with Jiang *et al.* (2014) where plasma-treated tomato plants had improved growth performances which leads them to readily fight against bacterial wilt caused by *Ralstonia Solanacearum* thus lowering their disease severity. This healthier growing condition likely contributes to bolstering their defence mechanisms and ultimately affects their capacity to withstand pathogen assaults.

Plasma treatment might enhance plant responses to pathogen through inactivation effect on the pathogen. Studies carried out on fungal infected *Philodendron erubescens* cv. Green Emerald leaves that was treated with plasma reported that free radicals released by plasma treatment can destroy the unsaturated fatty acids and protein molecules in cell membranes of pathogens. This will lead to severe membrane damages and subsequently to cell death of pathogens. It was found that oil vacuoles and their contents of fungal cells disappeared after plasma inactivation (Zhang *et al.*, 2014). Ostrikov *et al.* (2013) reported that targeted delivery of plasma-generated species can induce the desired apoptosis of pathogen cells. Plasma-generated species were found to effectively inactivate

Enterococcus faecalis bacteria in a few tens of seconds by penetrating as deep as 25 μM into their multilayer biofilm (Pei *et al.*, 2012). This suggests that reactive oxygen species (ROS) can pass through the microns-sized and epidermal pores of leaf cell membrane which causes high oxidative effects on pathogen cells in leaves. The O and OH radicals of ROS species can even penetrate through the cell membrane and react with polysaccharides, protein molecules and oil vacuoles which will lead to pathogen cell inactivation (Zhang *et al.*, 2014).

Significant increase in the production of H_2O_2 during the first 24 h following *Ralstonia solanacearum* inoculation (bacterial wilt) was observed in plasma-treated tomato plants compared to controls without plasma treatment which leads to reduced disease severity (Jiang *et al.*, 2014). This reveals that plasma treatment significantly increases the production of H_2O_2 following infection which helps plants to readily fight against pathogen. This is because H_2O_2 is an important ROS produced during early infection stages where it plays crucial role in plant disease resistance by serving as a signal to turn on the natural defence mechanism. This supports our findings where ROS which might have produced following CP treatment might have led to inactivation or slow down the progression of the *Foc* TR4 fungal pathogen leading to improved disease tolerance in 'Berangan' plants as observed in section 4.4 and 4.5. However, it is also important to consider that, in our case CP treatment was targeted at the corm of 'Berangan' plants and not leaf which might have influenced the plasma effects on banana plants. Thus, there are many aspects that need to be studied and optimized in the future that may increase the tolerance trait of 'Berangan' plants against *Foc* TR4. Due to several limitations including number of plant materials that can be treated with plasma at a time, space for growth and time, only one plasma treatment (with no variation of treatment time and strength) can be carried out.

5.5 Expression analysis of defence and growth-related genes

In addition to growth, disease progression and disease severity observation, the expression analysis of *Foc*-responsive growth and defence-related genes are important to further support the validation of progressive *Foc* TR4 infection in plasma-treated plants. Since, it is not fully understood on how CP treatment may improve the agronomical traits such as growth and disease tolerance in ‘Berangan’ plants, expression study was conducted using growth and defence-related genes to get some insights on the molecular effects of CP treatment on the ‘Berangan’ plants.

5.5.1 Validation of designed primers for growth and defence-related genes

In the process of designing primers for targeted growth and defence-related genes in gene expression analysis, ‘DH Pahang’ was selected as the reference sequence. This selection was made due to the unavailability of ‘Berangan’ cultivar sequence information in any databases. Moreover, ‘DH Pahang’ serves as a suitable candidate to be used as reference gene as both ‘Berangan’ and ‘DH Pahang’ share an A genome composition, with ‘Berangan’ and ‘DH Pahang’ are made of AAA and AA genome, respectively. Primer pairs that are suitable for all the downstream application should obey the allowed primer parameters, such as melting temperature (50 - 65 °C), GC content (40 – 60 %), length of primers (18 – 22 bp) and absence of secondary structures (hairpin, GC clamp, primer dimer, self-annealing) (Singh *et al.*, 2001; Borah, 2011). Based on the suitability test done using SMS software, all the primers designed passed most of the primer parameters (Appendix B). However, hairpin formation (*PRI*), self-annealing (*PRI*, *CEBiP*, *ChiH*, *PAL*, *Cytochrome P450*) and GC clamp (*Cytochrome P450*) were encountered in one of the primers from designed primer pairs and was unavoidable. Thus, to further verify the reliability of these primer pairs, primer specificity test was done to check the primer efficiency. PCR primer efficiency should be between 90 % – 110 % indicating that the amount of amplified product doubles perfectly during each cycle.

Referring to Table 4.4, all the primers designed have efficiencies between 90 % – 110 % indicating their reliability as well as comparability and thus can be used for downstream applications (Ramakers *et al.*, 2003). Single peak observed in melt curve plot analysis also indicates that the amplified products are single discrete species (Figure 4.12) (Li *et al.*, 2003). Furthermore, the specificity of designed primers was also verified using PCR and sequence analysis. Single band with expected band size (~100 bp) was obtained for all the designed primers in PCR followed by AGE indicating that only one specific product was amplified (Figure 4.7). The identity of the amplified product was further verified using the sequence analysis. The sequencing results obtained were blasted using Phytozome and NCBI database. The results obtained showed highest percentage of similarity with the targeted product which further verified that the designed primer pairs are specific and suitable for downstream applications (Figure 4.8 and 4.9).

5.5.2 Gene expression analysis

This present study aimed to investigate the potential effect of plasma on expression of several *Foc* TR4-responsive genes including defence-related (*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*) and growth-related (*Cytochrome P450*, *NAC68*, *CAT*) genes. The detailed analysis of the gene expression profiles provides valuable insights into the possible mechanisms by which the plasma-treated plants displayed slower disease progression and slightly lower disease symptoms.

The expression analysis revealed a significant trend where most of the chosen genes (except *ChiH*) exhibited heightened expression primarily in plasma-treated plants after *Foc* inoculation. This intriguing observation implies that plasma might play a crucial role in enhancing plant responses against various stresses, particularly when faced with *Foc* attack.

A defence-related gene, *PR1*, is a member of the PR family proteins. *PR1* is a dominant group with antifungal activity which will be induced by pathogens or salicylic acid. Study reported that the transcript level of *PR1* increased as a response to *Foc* TR4 attack despite banana genotypes (Li *et al.*, 2015; Zhang *et al.*, 2019). In this study, gene expression profile of *PR1* showed that it was upregulated and showed higher expression in *Foc* inoculated plants, both in the plasma-treated (Group 1) and untreated (Group 2) plants compared to non-*Foc* inoculated plants (Group 3) over time within 72 hours post *Foc* TR4 infection. This observation was in accordance with Van de Berg *et al.* (2007) which showed that tolerance in banana against *Fusarium* wilt is associated with the early up-regulation of cell wall-strengthening genes, *PR1* in the roots. As for *Foc* inoculated plants untreated with plasma (Group 2), *PR1* expression was found to be the highest at 24 hpi and decreased afterwards. This was in accordance with the study by Li *et al.* (2017) where in plants inoculated with *Foc* TR4, *PR1* expression was found to decrease in expression after 24 hpi. This suggests that *PR1* gene is being induced highly during very early stages of infection and responsible for the early defence response. However, in *Foc* inoculated plants that had been treated with plasma (Group 1), the *PR1* expression does not reduce at 72 hpi but peaked with a significant increase (63-fold change) which may explain the phenotypic observation in the challenge experiment (bioassay) (section 4.4 and 4.5) where plasma-treated plants showed 1-week delayed disease progression and had lower DSI. It is possible that higher *PR1* gene expression in the plasma-treated plants facilitate in the better tolerance against *Foc* infection since PR proteins are crucial in plant innate immunity (Niu *et al.*, 2018). The upregulation of *PR1* observed in *Foc* inoculated plants over time until 72 hpi suggests that *PR1* encoded proteins are responsible for plant defence in banana roots.

Another defence-related element, WRKY transcription factors (TFs), play crucial role in the regulation of plant growth, development, and response towards variety of abiotic

and biotic stressors (Jia *et al.*, 2022). In this study, the expression of two WRKY genes namely *WRKY22* and *WRKY50* was evaluated. *WRKY22* gene belonging to the WRKY TF family plays an important role in plant resistance against biotic stress. In the present study, *WRKY22* was upregulated with higher expression in *Foc* inoculated plants (Group 1 and 2) compared to non-*Foc* inoculated plants (Group 3) from 24 hours post *Foc* TR4 infection. This observation further supports the role of *WRKY22* in early defence response mechanism. This was in accordance with Sun *et al.* (2019) and Zhang *et al.* (2019) where *WRKY22* was found to be upregulated in both resistant and susceptible banana plants following *Foc* TR4 attack. As for *Foc* inoculated plants that were untreated with plasma (Group 2), the expression of *WRKY22* increased from 24 hpi but slightly decreased in expression at 72 hpi. This result was in accordance with Sun *et al.* (2019) where the expression of *WRKY22* started to decrease after 48 hpi. However, significant increase (18-fold change) of *WRKY22* expression was observed after 48 hpi in plasma-treated plants inoculated with *Foc* (Group 1) at 72 hpi compared to the plants from other treatment groups. This result suggests that plasma treatment may influence upregulation of *WRKY22* similarly to *PR1* expression during the early *Foc* TR4 infection stages which ultimately contributing to the improved disease severity and disease tolerance observed in section 4.4 and 4.5. As for the non-*Foc* inoculated plants (Group 3), it is crucial to notice that *WRKY22* was found to maintain upregulation at 2 hpi and 24 hpi which then downregulated afterwards. This suggests that plasma may induce *WRKY22* upregulation even without pathogen attack. This finding might be in accordance with Zhang *et al.* (2019), where the *WRKY* genes were reported to be involved in constitutive defence mechanism even without infection. This upregulation observed in group 3 might also be due to plasma treatment as observed in group 1. However, the *WRKY22* expression downregulated at 48 hpi and 72 hpi suggesting that plasma treatment alone is not sufficient for the upregulation of *WRKY22* gene.

Similarly, *WRKY50* gene demonstrated substantial expression and peaked in *Foc* inoculated plants that underwent plasma treatment (Group 1) at 72 hpi. Nevertheless, the elevated gene expression never surpassed that of the untreated plants (Group 2). This distinction highlights that plasma treatment did not exert a significant influence on *WRKY50* expression. However, the observations in *Foc* inoculated plants further underscore the role of *WRKY50* in plant defence against *Foc* TR4 attack. Notably, it is important to notice that at very early hours of infection, *WRKY50* expression was not upregulated in *Foc* inoculated plants untreated with plasma (Group 2) which was in accordance with a study by Pinheiro *et al.* (2022). As for the non-*Foc* inoculated plants treated with plasma (Group 3), no significant change observed indicates that *WRKY50* gene is triggered by both pathogen attack and plasma treatment and not by individual factor (plasma).

The next defence-related gene is *PAL*. The *PAL* gene, a vital enzyme in the phenylpropanoid pathway, governs the synthesis of phenolic compounds and phytoalexins with antimicrobial properties (Yadav *et al.*, 2020). Elevated expression of *PAL* genes was observed following biotic stress exposure (Giberti *et al.*, 2012; Zhang *et al.*, 2019). In the present study, the *PAL* expression was upregulated in *Foc* inoculated plants (Group 1 & 2) and notably, this upregulation occurred earlier in plasma-treated plants compared to their untreated counterparts, regardless of *Foc* inoculation. The expression of *PAL* was only increased and peaked at 48 hpi in plants untreated with plasma and *Foc* inoculated (Group 2). This finding is in accordance with Mohd-Yusuf *et al.* (2019), as same pattern of *PAL* gene expression was reported where the expression was highest at 48 hpi and decreased afterwards following *Foc* R4 attack in ‘Berangan’ plants. Similar pattern was also reported in Li *et al.* (2013), where the expression at very early stage (3hpi) was downregulated, upregulated at 27 hpi and reduced afterwards at 51 hpi in susceptible Cavendish cultivar following *Foc* TR4 attack. The upregulation of *PAL*

in plasma-treated plants regardless the *Foc* inoculation (Group 1 and 3), indicates the involvement of *PAL* induction beyond pathogen attack, potentially attributed to the effects of plasma treatment.

Two genes associated with pathogen-triggered immunity were also analysed in this study, namely *CEBiP* and *ChiH*. The first gene, chitin elicitor binding protein (CEBiP) is a critical component in the plant signalling pathway which recognizes and degrades the chitin oligosaccharides of fungal pathogen (Chen and Ronald, 2011). The gene was reported to be highly expressed in resistant banana cultivar compared to susceptible banana cultivar following *Foc* TR4 attack (Thangavelu *et al.*, 2020). *CEBiP* was found to be upregulated at the very early stage of *Foc* inoculation (2 hpi) in plasma-treated plants (Group 1). The untreated plants (Group 2) only displayed late upregulation of the gene at 72 hpi. No upregulation of *CEBiP* gene was observed at all timepoints in non-*Foc* inoculated plants treated with plasma (Group 3). This indicates that the upregulation of *CEBiP* mainly depends on pathogen (*Foc* TR4) attack and not plasma. This might be due to the induction of plant defence response by the presence of chitin oligosaccharides which serves as pathogen elicitors to trigger PAMP-triggered immunity (PTI) (De Jonge *et al.*, 2010). The upregulation of *CEBiP* in *Foc* inoculated plants untreated with plasma (Group 2), at 72 hpi is in accordance with few studies (Bai *et al.*, 2013, Li *et al.*, 2012). These studies reported late induction of *CEBiP* which was at 5- and 10-days post inoculation of *Foc* TR4 in susceptible banana cultivars.

The second gene involved in pathogen-triggered immunity examined in this study is *ChiH* gene. *ChiH* encodes for chitinase enzyme which is crucial for fungal growth inhibition induced by plant hormone ethylene and pathogen attack. In the present study, *ChiH* was found to be upregulated only in *Foc* inoculated plants untreated with plasma (Group 2) at 72 hpi. It is evident that plasma had no discernible effect on the expression of *ChiH* gene. The late upregulation of *ChiH* is in accordance with Zhang *et al.* (2019)

and Bai *et al.* (2013) where chitinase enzyme was upregulated especially during the later infection stages. However, the reason behind the lack of *ChiH* upregulation in plasma-treated plants after *Foc* inoculation remains unclear.

In addition to defence-related genes, several growth-related genes were also examined in this study including *Cytochrome P450*, *NAC68* and *CAT*. These genes exhibited complex patterns of expression influenced by both plasma treatment and *Foc* TR4 infection. The significant upregulation of *Cytochrome P450* in plasma-treated + *Foc* inoculated plants (Group 1) suggests that plasma treatment enhances the defence response by inducing this gene. Cytochrome P450s are known to be the multifunctional heme-thiolate proteins that are responsible for growth and defence in plants (Pandian *et al.*, 2020). Wang *et al.* (2012) and Zhang *et al.* (2019) reported the upregulation of *Cytochrome P450* following *Foc* TR4 attack in banana plants. Plasma treatment was shown to induce upregulation of the gene at early stage (starting from 24 hpi) in association with *Foc* TR4 infection. It is plausible that plasma treatment elevated the expression of *Cytochrome P450*. Ultimately, the elevation might have a cumulative effect that enhances the plant's response to pathogens (delayed disease progression and lower DSI value). This could be linked to the role of the gene in lignin synthesis and production of defence compounds as reported by Dong *et al.* (2020).

Similarly, *NAC* demonstrated differential expression patterns influenced by both plasma and *Foc* TR4 infection, highlighting their role in mediating responses to both stressors. *NAC*, plant-specific TF is involved in the regulation of plant development and stress (Niu *et al.*, 2018). *NAC* plays an important role in the regulation of secondary wall deposition (Negi *et al.*, 2019). In this present study, it was found that in *Foc* inoculated plants untreated with plasma (Group 2) the expression of *NAC68* gene was upregulated following *Foc* TR4 inoculation from 24 hpi till 48 hpi. This shows the role of *NAC68* in plant defence against *Foc* TR4 attack. However, at 72 hpi the expression of *NAC68* was

downregulated in *Foc* inoculated plants untreated with plasma (Group 2) but upregulated in plasma-treated plants (Group 1) indicating the role of plasma in inducing *NAC68* gene. Considering non-*Foc* inoculated plants treated with plasma (Group 3) that showed no significant upregulation of *NAC68*, it is evident that the induction of *NAC68* expression is not solely attributed to plasma treatment alone. In *Foc* inoculated plants, plasma-treated plants (Group 1) showed better expression compared to plasma untreated plants (Group 2) indicating that *NAC68* which is a stress responsive gene is being highly induced by the extra stress caused by plasma treatment such as release of ROS besides *Foc* TR4 attack. This is further supported by the upregulation observed in non-*Foc* inoculated plants treated with plasma (Group 3) at 2 hpi (early infection). This implies that plasma treatment might induce a stress response that subsequently triggers the induction of *NAC68* gene.

The final gene examined is *Catalase* (*CAT*). This gene encodes an antioxidant enzyme which functions as a ROS scavenger, involves in ROS detoxification which helps in minimizing the host cell damage caused by ROS (Mittler *et al.*, 2004; Kapoor *et al.*, 2019). In the present study, *CAT* gene expression was found to be highly expressed in plasma-treated plants following *Foc* inoculation. This result is in accordance with Mujahid *et al.* (2020) where *CAT* expression reported to be higher in plasma-treated grapes plants at all timepoints after plasma treatment (2, 8 and 14 days after treatment). It is important to note that plasma treatment alone was not able to elevate the expression of the gene compared to combination effect of plasma treatment and *Foc* inoculation. This shows that both plasma and *Foc* works together in inducing *CAT* gene. The presence of *CAT* enzyme, responsible for ROS scavenging, likely contributes to improved plant health while minimizing cellular damage.

Overall, all the defence genes selected for the gene expression profile study (*PRI*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP* and *ChiH*) were found to be involved in the ‘Berangan’

plant defence against *Foc* TR4 infection as all of them are differentially expressed upon *Foc* TR4 attack. Interestingly, the gene expression study offers valuable insights on the possible mechanisms through which plasma could enhance plants' ability to combat diseases in particular by *Foc*. As for the plasma treatment, all the defence genes except for *ChiH* were found to be differentially induced and upregulated in plasma-treated plants. Meanwhile, the growth genes (*Cytochrome P450*, *NAC68* and *CAT*) were found to be induced differentially by both plasma treatment and *Foc* TR4 infection.

The study revealed the connection between growth and defence-related genes, underscoring their collaboration in the plant's response to *Foc* TR4 infection and plasma treatment. Plasma treatment appeared to synergize with *Foc* TR4 infection in increasing the expression of key genes involved in both growth and defence mechanisms contributing to the improved growth and disease tolerance observed in the phenotypic assays. Enhancement of growth genes following plasma treatment modestly enhanced banana plant growth improving their general health. Besides, enhanced expression of defence genes following plasma treatment also contributed to delayed disease progression suggesting that plants might be able to tolerate the *Foc* attack better by slowing down their infection progression. Improved plant health and ability to tolerate *Foc* TR4 attack better as a result of growth and defence gene expression enhancement following plasma treatment contributed to improved banana plants' readiness to fight better against pathogen attack (*Foc* TR4).

Taken together, our findings suggest that the plasma treatment may be an important inducer of the plant defence in response to *Foc* TR4 attack. Besides, our finding also suggests that plasma treatment alone does not significantly induce the *Foc*-responsive genes but together with the induction caused by pathogen, *Foc* TR4. This might be due to the extra stresses caused by both plasma treatment. This is because Adhikari *et al.* (2020) reported that the ROS released by cold plasma priming alters epigenetic regulation

in plant seeds that further modulates stress-related genes which leads to tomato plant improvement. This suggests that plasma treatment might be able to trigger plant tolerance to pathogens by activating systemic immune responses in plants even before pathogenic encounters due to the plasma induced stress, subsequently triggering defence mechanisms. In conjunction with stress caused by *Foc*, the heightened expression of specific defence and growth-related genes could collectively contribute to delaying disease progression and potentially reducing DSI values.

It is also important to note that since the growth observation is independent from *Foc* infection, plasma treatment alone might have induced the growth genes earlier right after CP treatment which leads to favourable growth observed in section 4.3. This agrees with upregulations of growth genes observed in non-*Foc* inoculated plants treated with plasma (Group 3). This might be because the growth genes would have been induced even more earlier right after CP treatment and reduced in expression at later stages (*Foc* challenge). Findings from gene expression profile suggest that plasma treatment has the potential to activate plant immunity by enhancing key genes involved in growth and defence, enabling plasma-treated plants to thrive more effectively in the presence of pathogens.

5.6 Future study and limitations

The present study restricted by several limitations despite the valuable insights provided which must be taken into consideration for improvement in future studies. Firstly, (cold) plasma parameter optimization needs to be done to select the best parameters which can provide significant effect in improving disease tolerance in banana plants. To date, there is no available study on the suitable plasma parameter that could improve traits in tissue culture derived banana plants. Since, there is no information available on the survival rate of tissue culture plantlets following plasma treatment, plasma parameter with no variation in duration and strength was used in this study to explore the use of CP treatment on tissue culture banana plantlets. The CP setup which is the plasma jet used in this study leads to spatial constraints which limited the number of plantlets that can be treated with plasma at a time. Thus, other setups and methods of CP treatment such as the use of corona discharges, dielectric barrier, plasma activated water should also be explored to select the best CP treatment approach that could improve banana defence response in combating Fusarium wilt.

Next, the number of plants used in the present study are small due to few factors such as CP setup that can only fit one plant at a time, spatial constraints for growth and time limitations. The fragility of tissue culture plantlets further compounds this challenge. Thus, only a smaller number of plants were involved in this study. To enhance the robustness of findings, future investigations should endeavour to involve a larger number of banana plants, thus facilitating a more comprehensive examination of the effects of plasma treatment.

Additionally, this study lacks the knowledge on the expression profile of middle and late defence response genes as it only focused on the early defence response genes which can reduce the severity of Fusarium wilt. Studying the overall expression profile at different timepoints are crucial for better understanding of the mechanisms involved in

the plasma treatment that could influence the banana defence response against *Fusarium* wilt. Furthermore, other omics studies should be done such as proteomics and metabolomics to investigate the change in the response of *M. acuminata* cv. 'Berangan' towards *Foc* TR4 due to plasma treatment. This will provide an insight on the metabolites and proteins triggered by plasma treatment which is very crucial in the successful containment of *Fusarium* wilt.

Even though the present study offers valuable insights, it also points to various avenues for improvement in future research endeavours. Overcoming the outlined limitations will undoubtedly contribute to a more comprehensive and better understanding of the interplay between CP treatment, gene expression, and banana's defence response against *Foc*.

CHAPTER 6: CONCLUSION

This present study demonstrated the potential of cold plasma (CP) treatment as a valuable tool to be utilised in combating Fusarium wilt disease in banana plants. In this study, cold plasma treatment (CP) was shown to positively influence the growth of *M. acuminata* cv. 'Berangan' plants, as evidenced by slight improvements in growth parameters such as height, number of leaf and stem diameter in plasma-treated banana plants compared to control plants. Additionally, plasma treatment was found to delay the progression of Fusarium wilt disease by one week and reduce disease severity following *Foc* TR4 inoculation in comparison to untreated plants. However, it is important to note that plasma-treatment does not completely makes the plants confer resistance against Fusarium wilt caused by *Foc* TR4. These phenotypic observations aligned with the gene expression profile of all the early defence and growth-related genes studied. Majority of the selected defence-related genes (*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*) and all three selected growth-related genes (*Cytochrome P450*, *NAC68*, *CAT*) genes were found to be differentially upregulated and expressed due to CP treatment followed by *Foc* TR4 inoculation. Plasma treatment alone does not provide significant induction of *Foc* TR4-responsive genes (defence and growth) but together with *Foc* TR4 inoculation.

This study's gene expression profiles offer invaluable resources for future investigations aimed at deeper comprehension of the molecular mechanisms underpinning resistance to Fusarium wilt disease caused that can possibly be induced by plasma treatment. Such insights can potentially pave the way for potential utilization of CP as an alternative effective approach in Fusarium wilt management, thereby reducing disease severity, and stalling its progression through growth enhancement. The delayed disease progression observed in this study holds promise for farmers seeking to maximize their banana yields and profits, thereby contributing to food security and economic improvement.

In conclusion, cold plasma treatment has been shown to exert a positive influence on both growth and enhanced tolerance against Fusarium wilt disease caused by *Foc* TR4. Nonetheless, further research is necessary to optimize treatment parameters for maximizing the desired effects.

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