POTENTIAL OF UTILIZING BIOLOGICAL AND CHEMICAL AGENTS IN THE CONTROL OF FUSARIUM WILT OF BANANA

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POTENTIAL OF UTILIZING BIOLOGICAL AND CHEMICAL AGENTS IN THE CONTROL OF FUSARIUM WILT OF BANANA

by

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I hope this thesis has great benefits for the knowledge generally in agriculture and especially in plant pathology, the pathogenic *Fusarium oxysporum* f.sp. *cubense* TR4 causing wilts disease symptoms on banana and control without the chemical pesticides, which is a detrimental effect on the environment.

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LIST OF ABBREVIATIONS

®	Registered identity assigned to a product
ºC	Degree Celsius
%	Percentage
50% W.G.	Active ingredient 50 g of total *weight* of commercial product
ß-tubulin	ß-tubulin primer
Actigard™	BION®, trade name in United States of America
ANOVA	Analysis of variance
Actigard™	BION®, trade name in United States of America
ANOVA	Analysis of variance
AP	Post-inoculation, added pathogen before treatment with factor
ASM	Acibenzolar-S-methyl
BCAs	Biological control agents
BION®	Benzo (1, 2, 3) thiadiazole-7-carbothioic acid-S-methyl ester
BLAST	Basic Local Alignment Search Tool
BLOCKADE®	BION®, trade name in other world
BOOST®	BION®, trade name in other world
bp	Base pair
CFU	Colony Forming Unit
CLA	Carnation Leaf-piece Agar
cm	Centimeter
CMV	Cucumber mosaic virus
DNA	Deoxyribonucleic acid

ddH_2O	Deionized distilled water
ech42	Chitinase primer
EF	Endophyte *Fusarium*
e.g.	For example
et al.	And other
FAO	Food and Agriculture Organization
FO	*Fusarium oxysporum*
Foc	*Fusarium oxysporum* f.sp. *cubense*
*Foc*TR4	*Fusarium oxysporum* f.sp. *cubense* Tropical Race 4
Fol	*Fusarium oxysporum* f. sp. *lycopersici*
Fod	*Fusarium oxysporum* f. sp. *dianthi*
f.sp.	Formae specialis
Fusarium-ID	FID
GenBank	G.B.
h	Hour
HSD	Tukey's Studentized range test
ISR	Induced systemic resistance
JA	Jasmonates
kg/cm2	kilogram-force / square centimetre
MEGA V5.1	Molecular Evolution Genetic Analysis version 5.1
MeJA	Methyl jasmonate
min	Minute
ml	Millilitre
mm	Millimetre

mRNA	Messenger ribonucleic acid
N	Nitrogen
npF	Non-pathogenic *Fusarium* spp. include *Fusarium oxysporum* (Avirulent and low virulent), and *Fusarium* spp. associated of healthy banana tree
ng	Nanogram
PAL	Phenylalanine ammonia lyase
PCR	Polymerase Chain Reaction
PDA	Potato-dextrose agar
POX	Peroxidases
PPO	Polyphenol oxidase
PPA	Peptone pentachloronitrobenzene agar
PR	Pathogenesis related proteins
RBA	Rose bengal agar
rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolutions per minute
SA	Salicylic acid
SAR	Systemic acquired resistance
s	Second
sp.	Species
SP	Spilt-root inoculation, add factor together with pathogen in separate root
spp.	Several species
T	ß-tubulin primer

TEF-1α	EF primer - α-elongation factor
tef1	TEF primer of Trichoderma spp. - α-elongation factor
TR4	Tropical root race 4
µg	Microgram
µl	Microlitre
µm	Micrometer
USM	Universiti Sains Malaysia
UV	Ultra violet
V	Volt
VCG	Vegetative compatibility group
WA	Water agar

KEUPAYAAN PENGGUNAAN AGEN BIOLOGI DAN KIMIA DALAM PENGAWALAN LAYU FUSARIUM PADA PISANG

ABSTRAK

Satu kelompok ras baru patogen dikenali sebagai ras 4 tropika (*Foc*TR4) adalah paling virulen dan penyebab penyakit layu pisang di Asia Tenggara, termasuk Malaysia. Langkah-langkah kawalan semasa termasuk penggunaan racun kulat dan kultivar rintang serta pencegahan kemasukan penyakit ini ke kawasan baru tidak menunjukkan kesan memberangsangkan. Kajian ini bertujuan untuk meneroka kemungkinan menggunakan agen biologi untuk mengawal penyakit ini iaitu *Fusarium* dan *Trichoderma*. bukan patogen dan juga bahan kimia perangsang iaitu BION® (Acibenzolar - S - methyl). Lima puluh satu pencilan *Fusarium* (43 *F. oxysporum* dan lapan *Fusarium* spp.) serta 31 *Trichoderma* telah dipencilkan daripada pokok pisang dan tanah rizosfera. Semua pencilan dicamkan berdasarkan morfologi dan urutan *TEF-1α* dan *ß-tubulin* (*Fusarium*) dan *Tef1* dan *ech4* (*Trichoderma*). Dua puluh tujuh pencilan dikenalpasti sebagai *Foc*TR4 menggunakan primer khusus. Daripada ujian kepatogenan, tujuh pencilan adalah bukan patogenik manakala 36 pencilan adalah patogenik. Pencilan LJ27 adalah yang paling virulen, maka, ia digunakan sebagai faktor patogenik untuk semua eksperimen.. Empat pencilan npF, LJ20 (*F. fujikuroi*), 13v1 (*Foc*TR4), 6p1 (*F. solani*) dan 1322 (*F. oxysporum*) berkesan mengurangkan keterukan penyakit dan dipilih untuk digunakan di dalam eksperimen selanjutnya menggunakan kaedah inokulasi campuran, inokulasi pemisahan akar and pasca-inokulasi. Pencilan ini sangat berkesan mengurangkan penyakit menggunakan kaedah inokulasi pemisahan akar. Kesan penindasan juga telah diperhatikan di dalam ujian kultur dual pencilan *Trichoderma* melawan LJ27. Empat pencilan, Tveg1 (*T.*

harzianum), TL5 (*T. harzianum*), T26 (*T. parareseii*) dan TR102 (*T. koningii*) berjaya mengurangkan penyakit kepada 0% dan dipilih untuk kajian selanjutnya. Penilaian *in situ* menunjukkan pencilan-pencilan ini mengurangkan insiden penyakit kepada 0% di dalam kaedah pra-inokulasi. Penilaian *in vitro* sebatian kimia peransang, BION® menggunakan empat kepekatan (0.8, 1.6, 2.6, and 4 µg/L) melawan LJ27 menunjukkan hanya sedikit kesan penindasan. Di dalam penilaian *in situ* kesemua kepekatan menunjukkan penindasan yang tinggi ke atas penyakit layu menggunakan kaedah pra-inokulasi dan inokulasi campuran. Berdasarkan kesegahan tumbuhan dan kajian histologi, pencilan npF, *Trichoderma* dan BION® berupaya meningkatkan pertumbuhan vegetatif pokok pisang. Pencilan npF menghasilkan 15 sebatian meruwap yang mungkin bersifat antikulat seperti Ethylbenzene, Hexadecanoic acid, 1-(hydroxymethyl)-1, 2-ethanediyl ester dan kaur-16-ene. Manakala, pencilan *Trichoderma* menghasilkan 30 sebatian meruwap seperti Butane, 1-(1-methylpropoxy), 2 (3H)-Furanone, dihydro-5-methyl- dan 2-Pyrrolidinone. Siderophores yang berperanan merencat pertumbuhan *Foc*TR4 dan meningkatkan pertumbuhan pokok juga dikesan. Kesimpulannya, kajian ini menunjukkan potensi *Fusarium* bukan patogenik dan *Trichoderma* sebagai agen kawalan biologi dan BION® sebagai agen kawalan kimia untuk mengawal *Foc*TR4.

POTENTIAL OF UTILIZING BIOLOGICAL AND CHEMICAL AGENTS IN THE CONTROL OF FUSARIUM WILT OF BANANA

ABSTRACT

A new race of the pathogen, known as tropical race 4 (*Foc*TR4), is the most virulent and the causal agent of Fusarium wilt disease in Southeast Asia including Malaysia. Current control measures include the use of fungicides and resistant cultivars, and preventing the introduction of the disease into new areas have not shown promising results. This study was aimed to explore the possibility of using biological agents to control the disease i.e. non-pathogenic *Fusarium* (npF) spp., *Trichoderma* spp. and a chemical inducer, BION® (Acibenzolar - S - methyl). Fifty one *Fusarium* isolates (43 *F. oxysporum*, eight *Fusarium* spp.) and 31 *Trichoderma* isolates were isolated from banana plants and rhizosphere soils. All isolates were identified using morphology and sequences of *TEF-1α* and *ß-tubulin* (*Fusarium*) and *tef1* and *ech42* (*Trichoderma*). Twenty seven isolates were confirmed as *Foc*TR4 using a specific primer. From pathogenicity test, seven isolates were non-pathogenic while 36 isolates were pathogenic. Isolate LJ27 was the most virulent, thus, was used as pathogenic factor in all experiments. Four npF isolates, LJ20 (*F. fujikuroi*), 13v1 (*Foc*TR4), 6p1 (*F. solani*) and 1322 (*F. oxysporum*) effectively reduced the disease, thus, were selected in subsequent tests using mixed, split-root and post-inoculation methods. The isolates effectively reduced the disease using a split-root inoculation method. The suppression effect was also observed in dual culture test of *Trichoderma* isolates against LJ27. Four isolates, Tveg1 (*T. harzianum*), TL5 (*T. harzianum*), T26 (*T. parareseii*) and TR102 (*T. koningii*) reduced the disease to 0% and were selected in subsequent tests. *In situ* evaluation showed the isolates reduced the disease

incidence to 0% in pre-inoculation method. *In vitro* evaluation of chemical inducer, BION® using four concentrations (0.8, 1.6, 2.6, and 4 µg/L) against LJ27 showed a slight suppression effect. In *in situ* evaluation, all concentrations highly supressed the disease in pre- and mixed inoculation methods. Based on plant vigour and histological studies, isolates of npF, *Trichoderma* and BION® were able to enhance the vegetative growth of banana plants. npF isolates produced 15 compounds possible with antifungal properties such as Ethylbenzene, Hexadecanoic acid, 1-(hydroxymethyl)-1, 2-ethanediyl ester and kaur-16-ene. *Trichoderma* isolates produced 30 different compounds such as Butane, 1-(1-methylpropoxy), 2 (3H)-Furanone, dihydro-5-methyl- and 2-Pyrrolidinone. Siderophores, which inhibits *Foc*TR4 growth and improves plant growth were also detected. In conclusions, the present study showed the potential of non-pathogenic *Fusarium* and *Trichoderma* as biological control agents and BION® as a chemical control agent to control *Foc*TR4.

CHAPTER 1

INTRODUCTION

The banana crop has been identified in the 3rd National Agriculture Policy (1998-2000) as one of the 15 most important fruit crops in Malaysia. Rohizad (1998) projected that fruit production would reach 422,784 metric tons by the year 2010. However, with the persistent occurrence of Fusarium wilt in many plantations, this lucrative industry is facing a bleak future.

Fusarium oxysporum Schlechtend. as amended by Snyder & Hansen: Fr., is a highly cosmopolitan organism that includes both pathogenic and non-pathogenic strains (Armstrong and Armstrong, 1975; Booth, 1977). Parasitic forms are recognized by their selective pathogenicity and designated as formae speciales (f. sp.) but these special forms cannot be separated based on a morphological basis (Nelson, 1990). A forma specialis (f. sp.) can be further sub-divided into pathogenic subgroups, called races of an isolate to certain cultivars of the host plant (Nelson, 1990). These races are determined on the basis of virulence to a set of differential host cultivars (Correll, 1991). The Fusarium wilt of banana is caused by the soil-borne fungal pathogen *F. oxysporum* f. sp. *cubense* (*Foc*). Fusarium wilt, also known as Panama wilt, causes a highly destructive disease on banana.

Four races of Fusarium wilt of banana have been reported, Race 1 individuals attack Gros Michel, Silk, Apple, Lady Finger and Latundan cultivars; race 2 attack Bluggoe and other plantains; and race 3 attacks *Heliconia* spp. (Su *et al.*, 1977). Before 1990, *Foc* was classified as the race 4 only of some isolates that caused serious losses in Cavendish genotypes in subtropical regions of Australia, the Canary Islands and Taiwan (Pegg *et al.*, 1996). The two subdivided of *Foc* race 4, viz. subtropical

race 4 (ST4) and tropical race 4 (TR4) were designated. Thus, a new variant of tropical race 4 of *Foc* that affects banana severely in the tropics was identified (Dita *et al.*, 2010). Also, it was found the TR4 isolates are pathogenic under both tropical and subtropical conditions (Buddenhagen, 2009). TR4 has caused severe damage to banana cultivars in Malaysia, Indonesia, South China, the Philippines, the Northern Territory of Australia, Africa, and Middle East (Ploetz, 2006a; Molina *et al.*, 2008; Buddenhagen, 2009; Garcia *et al.*, 2014; Ploetz *et al.*, 2015). Currently, PCR-based diagnostic tool was successfully exploited to be diagnostic of *Foc*TR4 by specific primer (Dita *et al.*, 2010).

Fusarium wilt could not be controlled effectively, since its discovery. The potential management of Fusarium wilt by various methods of control including fungicides (Lakshmanan *et al.*, 1987), crop rotation (Hwang, 1985; Su *et al.*, 1986), fumigation (Herbert and Marx, 1990); flood–fallowing (Stover, 1962a), and organic amendments (Stover, 1962a), and resistant cultivars (Moore *et al.*, 1999a). Planting of resistant varieties also cannot be implemented because these varieties are not usually preferred by consumers (Viljoen, 2002). Biological control of this disease has become popular, given its environment-friendly nature (Weller *et al.*, 2002; Fravel *et al.*, 2003). Many other groups of microorganisms have been proposed in the suppression of Fusarium wilts on other plants such as non-pathogenic *Fusarium* (Nel *et al.*, 2006a), *Trichoderma harzianum* strain TH-10 (Thangavelu *et al*., 2003), *Gliocladium* sp. (Nel *et al.*, 2006a), *Pseudomonas* spp. (Kloepper *et al.*, 1980; Larkin *et al.*, 1993), *Arthrobacter* spp. (Smith, 1977), Actinomycetes (Larkin *et al.*, 1993), *Bacillus* and *Clostridium* (Tu *et al.*, 1975).

Many isolates of non-pathogenic *F. oxysporum* derived from symptomless banana roots provided some degree of protection against *Foc* race 4 in the greenhouse

(Gerlach *et al.*, 1999). The nature of *F. oxysporum* itself, capable of surviving in plant (host) such as vascular tissues (pathogenic cycle), in soils and in moribund tissues (saprophytic cycle), further reduce the effectiveness of the applications to control the disease in the field. The populations of saprophytic *Fusarium* spp. are more diverse and reach to higher levels in suppressive soils of Fusarium wilt (Nel *et al.*, 2006b). Alabouvette and Couteaudier (1992) determined three modes of action on the efficiency of non-pathogenic *Fusarium* in biological control of Fusarium wilts as namely: competition for nutrients in the soil and rhizosphere (Alabouvette, 1990), competition for infection sites on the root surfaces (Nagao *et al.*, 1990), and induced resistance within the host (Mandeel and Baker, 1991).

On the other hand, Biological control of soil-borne diseases by *Trichoderma* spp. is well documented (Sivan and Chet, 1986). Many reports have indicated that *Trichoderma* spp. can suppress Fusarium wilt pathogens effectively (Calvet *et al.*, 1990) including Fusarium wilt of banana (Kidane and Laing, 2010). On the other hand, the biocontrol mechanisms of *Trichoderma* can be divided into mycoparasitism, competition, antibiosis, induced resistance, and action of cell wall degrading enzymes (Benítez *et al.*, 2004).

Plants generally have the capability to activate their own defence mechanisms against attack by plant pathogens and pests (Kessmann *et al.*, 1994; Ryals *et al.*, 1994). It has been found that synthetic chemical compounds could naturally activate the systemic resistance that reflects responses in plants to protect them against pathogen attack (Kessmann *et al.*, 1994). The most thoroughly investigated chemical inducers known, commercially as BION® is the first commercially used synthetic activator of Systemic Acquired Resistance (SAR), and contain (Acibenzolar - S - methyl) (Oostendorp *et al.*, 2001). BION® imparts a protection to banana against *Foc*

race 4 (Moore *et al.*, 1999b). The mechanisms of disease suppression on Fusarium wilt of banana are through induction of host defences, direct antagonism towards *Foc* as well as increased plant vigour. Hence, structural features of a healthy plant and structural modifications occurring in response to infection may help both to exclude pathogen from the vascular system and to limit its spread within the system of the plant (Beckman and Talboys, 1981).

1.1. Objectives of study

1. To isolate and identify non-pathogenic *Fusarium oxysporum* and *Trichoderma* spp. from banana plants of specific cultivar (Berangan cv. Intan) in Malaysia.
2. To evaluate the efficiency of non-pathogenic *Fusarium* spp. and *Trichoderma* spp. in suppressing Fusarium wilt diseases incidence.
3. To assess some beneficial effects of *Foc* and incidence on banana plant.
4. To evaluate the influence of BION®, a chemical inducer for possibility of reducing Fusarium wilt disease incidence under greenhouse condition.

CHAPTER 2

LITERATURE REVIEW

2.1 The banana

Modern banana and plantains originated in Southeast Asia and Western Pacific regions where their inedible, seed-bearing, diploid ancestors can still be found in the natural forest vegetation (Pillay and Tenkouano, 2011). The banana is one of the most important fruit crops in the world, including Malaysia. The banana was cultivated mainly by smallholders (Köberl *et al.*, 2015). Edible triploid banana in Southeast Asia was further selected according to the vigour, fruit size and adaptability, and were developed at the expense of the original diploid types which is more inferior.

Banana is a large monocotyledonous herb (Simmonds and Shepherd, 1955) that belongs to the genus *Musa* (Family: *Musaceae*). It consists of an underground true stem, rhizome, and an above ground trunk which composed of tightly packed leaves sheath bases, known as the pseudostem. While there are about 40 species of *Musa* recognised (Jones, 1999), those with edible banana fruit originated from various combinations of just two species, *M. acuminata* Colla and *M. balbisiana* Colla (Simmonds and Shepherd, 1955). By convention, the haploid genome of *M. acuminata* is represented by 'A' and *M. balbisiana* by 'B'. Millennia of diploid hybridisation, diversification and human selection have resulted in three general groups of edible banana; the dessert banana (AA, AAA and AAB), cooking banana (ABB), and plantain (AAB) (Simmonds and Shepherd, 1955). There are also some

naturally occurring tetraploid bananas (AAAA), which are less common varieties of the wild banana *M. acuminata* (Simmonds and Shepherd, 1955).

2.2 Banana in Malaysia

In Malaysia, banana is the second most widely cultivated fruit, covering about 30,000 ha with a total production of 530,000 metric tons (mt). About 50% of the banana growing land is cultivated with Pisang Berangan and the Cavendish type. The popular dessert cultivars are Pisang Mas, Pisang Berangan, Pisang Rastali, Pisang Embun and Pisang Cavendish; while the popular cooking cultivars are Pisang Nangka, Pisang Raja, Pisang Awak, Pisang Abu and Pisang Tanduk (plantain). Traditionally, banana is planted as a cash crop or temporarily intercropped with oil palm, rubber and other perennial crops. There are only a few large banana plantations in Malaysia (Hassan, 2004). The Third National Agricultural Policy of Malaysia (1998 – 2010) listed banana as one of the 15 fruit crops ranked for commercial cultivation. Banana remains the second most important fruit crop (after durian), amounting to about 10 -12% of the total acreage under fruits (Masdek, 2003). This translates to the 30,000 hectares grown with banana and the acreage has somewhat stabilized over the past 10 years (1992 – 2001). Annual production has been slightly above one-half million tones, mainly consumed domestically, and less than 10% is exported (Masdek, 2003) mainly to Singapore, Brunei, Hong Kong and the Middle East (Hassan, 2004).

2.3 Diseases of banana

Banana diseases limit the areas of banana production. Many types of plant diseases, whether caused by fungi, bacteria, nematodes, viruses, or phytoplasma, are more severe and cause more serious losses to banana grown in the tropics (Jeger *et*

al., 1995). The diseases include Fusarium wilts caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc),* anthracnose caused by *Colletotrichum coffeanum* (Zakaria *et al*., 2009), black Sigatoka caused by *Mycosphaerella fijiensis* (Ploetz *et al.*, 2003), Cordana leaf spot caused by *Cordana musae* associated with *N. musae* and *N. musicola* (Hernández-Restrepo *et al.*, 2015), leaf speckle caused by *Cladosporium* (Surridge *et al.*, 2003), bacterial wilts caused by *Ralstonia solanacearum* (Meng, 2013), eight species of nematodes affecting banana (Masdek, 2003), banana streak disease caused by a *banana streak virus* (Harper *et al*., 2005; Gayral *et al*., 2010), bunchy top caused by *banana bunchy top virus* (Jeger *et al.*, 1995), and wilt disease caused by phytoplasma in the 16SrIV group (Davis *et al.*, 2012). However, until today, the most important disease of banana all over the world, including Malaysia, is Fusarium wilt caused by *Foc*, a soil-borne fungus. This disease is widespread and most of the commercial cultivars are very susceptible while the cooking cultivars are somewhat tolerant (Food and Agriculture Organization of the United Nations, 2010).

2.4 Fusarium wilt of banana

Fusarium wilt is caused by several formae speciales (f. spp.) of *F. oxysporum* and among the most severe diseases in many important crops around the world. In banana, the disease is caused by *F. oxysporum* f. sp. *cubense* (*Foc*). The overall banana production throughout the world has decreased due to the increasing threat of this disease, high labour costs, and marketing issues. The disease was first appeared in the Western Hemisphere towards the later part of the last century (Wardlaw, 1972).

Fusarium wilt disease was discovered in Australia in the late 1880, and it reached epidemic proportions in the 1950s. It destroyed 40,000 hectares of Gros Michel banana in Panama, and becoming a major threat to the banana industry in Central America. It took several years for scientists to identify the causative pathogen

that affected even the varieties that was thought to be disease resistant. However, the disease in Malaysia was different; it affected not only the Cavendish variety, but other variety was also infected more rapidly and was more deadly. Fusarium wilt was remained a major constraint to banana production worldwide (Ploetz *et al.*, 1992), and it recognized as one of the most widespread and destructive plant diseases in the recorded history of agriculture (Ploetz and Pegg, 1999).

2.4.1 Symptoms of Fusarium wilt of banana

Foc can infect banana at any stage. Once infected, the plant seldom reaches maturity because of a decline in growth and subsequent death of the plant. The first stage develops in the root tips of the plant at the small lateral or feeder roots (Stover, 1962a; Beckman, 1990). Then, the second stage after penetration takes place when the pathogen enters through wounds, the pathogen enters the xylem vessels and colonized the of vessel tissues (Sequeira *et al.*, 1958). In the third stage, the fungus invades the water conducting tissue (xylem), and produces microconidia that are transported to the plant, upper part thereby plugging the vascular tissues, and reducing the movement of water. Then, the fungal spores block the sieve cells of xylem, after that the spores germinated and spreads until blocking the whole tissues of xylem (Stover *et al.*, 1961; Jeger *et al.*, 1995). The internal symptoms of the Fusarium wilt of banana were visible dots of yellow, red or brownish and as streaks that are localized inside the vascular strands of the rhizome and pseudostem (Wardlaw, 1972). The discoloration of the rhizome is most severe when the stele joins the cortex (Stover, 1962a). In advanced stages of the infection, the discoloration of rhizomes is more obvious with intense pathogen growth.

The external symptoms observed in the banana show that the infection is typical of vascular wilt diseases. The symptoms of Fusarium wilt in banana consist of

yellowing in the oldest leaves or the lengthwise splitting of the lower leaf sheath (Ploetz, 2006a). The leaves begin to wilt and buckle at their petiole base, followed by the collapse of the leaves and death of the plant. The internal leaves will show brown streaks, which indicated the progress of the infection. Brown streaks can also be observed within older leaf sheaths. Xylem vessels also turn brick red to brown, as indication of fungus entry and its colonization on the rhizome and pseudostem which results in the blockage of the xylem tissues (Ploetz, 2006a).

2.4.2 Pathogen of Fusarium wilt

The causal organism of Fusarium wilt of banana is *F. oxysporum* a member of the section Elegans genus of a *Fusarium* (Fungi imperfecti). *Fusarium oxysporum*, as emended by Snyder and Hansen (1940), comprises all the species, varieties and forms, which were recognised by Wollenweber and Reinking (1935) in species description by Nelson (1981), within an intragenic grouping called section Elegans.

The described *F. oxysporum* was found to be a fungus transmitted through the soil (fungi soil -borne plant pathogen) (Booth, 1977). *F. oxysporum* pathogenic strains are well-known to be responsible for vascular wilt, crown rot, and root rot diseases in a wide range of economically important crops that comprise among other banana, oil palm, tomato, and asparagus (Baayen *et al.*, 2000). The basis of genetic host specificity (formae speciales) and cultivar specificity (pathogenic races) of *F. oxysporum* is unknown (Baayen *et al.*, 2000). Example of formae speciales including *Fusarium oxysporum* f. sp. *melonis* which only infected melons; *F. oxysporum* f. sp. *vasinfectum* infected cotton, and *F. oxysporum* f. sp. *cubense* is the causal agent of vascular wilt of banana.

For *Foc*, four races have been identified. Race 1 infected Gros Michel, Silk (AAB), Pome (AAB), Pisang Awak (ABB), Maqueno (AAB), and tetraploid 'I.C.2"

(AAAA) which was developed as a replacement for Gros Michel by the first banana breeding program in Trinidad. Race 2 is pathogenic on 'Bluggoe" and some bred tetraploids (AAAA) such as Bodles Altafort, a hybrid between Gros Michel and Pisang Lilin, which is resistant to race 1 (Ploetz, 2006b). Race 3 was reported to infect only *Heliconia* spp. and has mild effect on banana. Race 4 affects Cavendish cultivars, in addition to race 1- and 2- susceptible clones which include genotypes AAA, AAB, AA, ABB, and AAAA bred tetraploids (Stover, 1986).

Genotypically different tropical (T) and subtropical strains (ST) of race 4 have been recognised. Tropical race 4 (TR4) has caused severe damage to Cavendish cultivars in Malaysia, Indonesia, South China, the Philippines and the Northern Territory of Australia (Ploetz, 2006a) as well as Africa, and Middle East (Ploetz *et al.*, 2015) such as Jordan (Garcia *et al.*, 2014). PCR-based diagnostic tool have been developed to specifically detect the tropical race 4 (TR4), which is currently a major concern in global banana production.

Fusarium oxysporum is an anamorphic species that include the numerous plant pathogenic strains causing wilt diseases of a broad range of both agricultural and ornamental host plant (Appel and Gordon, 1996). Conidia are produced on monophialides and in sporodochia, and are dispersed loosely over the surface of mycelium (Griffin, 1994).

F. oxysporum produces three types of asexual spores: microconidia, macroconidia and chlamydospores (Nelson *et al.*, 1983). The characteristic of the species is by the longer conidia (especially in pionnotes) and extreme specialization as the cause of wilt disease of *Musa* sp. conidia in sporodochia and pionnotes, three-, seldom four-, exceptionally five-septate and the sizes are: 3-septate, 17 - 51 × 3 - 4.5 µm; 5 - septate, 36 - 57 × 3 - 4.7 µm (Gilman, 1959). The macroconidia are

multinucleate, germinate rapidly and are produced by the colony, thereby can proliferate this species efficiently. The macroconidia that can only be reliably examined either from the sporodochia or from the Carnation Leaf-piece Agar (CLA), as the shapes and sizes of the structure is more consistent and uniform when produced on CLA (Leslie and Summerell, 2006). Macroconidial primary characters observes are the shapes, sizes, number of septa, the shape of the apical- and basal cell and in most cases, a combination of all the characters which are sufficient for identification (Summerell *et al.*, 2003). Chlamydospores are viable, asexually produced accessory spores that are produced from the modification of structural vegetative hyphae segment(s) or from possessing the conidial cell thick wall, mainly consisting of newly synthesized material of cell wall (Schippers and van Eck, 1981). Chlamydospores have terminal/intercalary, globose or oval, one-celled or two - 7.25 µm, in mycelium, 5.5 - 9 µm. They also have sclerotia or sclerotial bodies that are blue-black, in a limited number, either 0.5 - 1 mm or up to 4 mm thick (Joseph, 1957). Both pathogenic and non-pathogenic *F. oxysporum* are morphologically indistinguishable from each other. Both somatic fusion and heterokaryon formation among individuals can occur separately of sexual reproduction, but usually only among strains of similar genotypes (Kistler, 1997).

The identification of *F. oxysporum* was based on morphological and molecular characteristics. Morphological characterization is based on the shapes of macroconidia and microconidia, structure of conidiophores and the presence of chlamydospores, either in singly, pairs or clumps (Beckman, 1987; Leslie and Summerell, 2006). PCR-based technique and PCR species-specific primers have been proven as a reliable diagnostic method for detection and identification of *Fusarium* species (Edwards *et al.*, 2002). The genes and regions that are extensively used in

DNA sequencing studies are *TEF-1α* and *β-tubulin* genes. The *TEF-1α* gene is the most common marker used for identification and characterization of *Fusarium* species (Geiser, 2004). *B-tubulin* gene has been used for identification of filamentous fungi, especially from plant pathogenic fungi such as *F. oxysporum* (Li and Yang, 2007). Partial DNA sequences of the *TEF-1α* gene (EF-1a) and *β-tubulin* gene exons and introns, were analysed to assess its phylogenetic relationship to the *Foc* and related Fusaria (Skovgaard *et al.*, 2003).

2.4.3 Life cycle and disease development of Fusarium wilt of banana

The life cycle of *F. oxysporum* starts with a saprophytic stage when the fungus survives in soil as chlamydospores (Beckman and Roberts, 1995). Chlamydospores that are dormant and static in the decayed plant tissue can be induced to germinate by nutrients that are secreted by extending roots of plants (Stover, 1962 a,b; Beckman and Roberts, 1995). After germination, the conidia are produced on the thallus within 6 - 8 hours, and chlamydospores in 2 - 3 days, but only when conditions are favourable. The infestation of the roots is followed by the penetration of the epidermal cells of a host or a non-host plant (Beckman and Roberts, 1995) and the development of a systemic vascular disease in host plants (Stover, 1970). With the advances phases of the disease, the fungus grows out of the vascular system to the neighbouring parenchyma cells, producing a great quantity of conidia and chlamydospores.

The chlamydospore formation and germination of pathogenic *Fusarium* species commonly takes place in the hyphae of both infected and decaying host tissues. Chlamydospore may also form abundantly from macroconidia that originate from sporodochia on the soil surface (Nash *et al.*, 1961; Christou and Snyder, 1962). Schippers and van Eck (1981) suggested that the formation of chlamydospore depends on the nutrient condition of the inocula. Once the carbohydrates are freed

from plant tissue or decaying roots, the chlamydospores start to germinate (Schippers and van Eck, 1981). Qureshi and Page (1970) further suggested that chlamydospores are formed by adding either organic or inorganic carbon sources.

It was also reported that there is a close similarity of chlamydospore formation in weak salt solutions on soil and in soil extracts. Hsu and Lockwood (1973) found that an environment deficient in energy, but with a suitably weak salt solution, may also infuse chlamydospore formation. Chlamydospore germination in nature seems to be dependent on exogenous energy sources (e.g. nitrogen and carbon) (Cook and Scroth, 1965; Griffin, 1969). Exogenous carbon and nitrogen were required for high or full chlamydospore germination at high spore densities (but not at low spore density) and in the soil (Cook and Schroth, 1965; Griffin, 1969; 1970). At low conidial concentrations, the conidia germinate, but do not convert into chlamydospores (Schneider and Seaman, 1974).

Infection: The infection caused by *F. oxysporum* in vascular tissues is complex and requires a series of highly regulated processes. First is adherence of which fungal infection begins when infection hyphae adhere to the host root surface (Bishop and Cooper, 1983a). Adhesion of fungi to the host surface is not a specific process, as they can adhere to the surface of both host and non-hosts (Vidhyasekaran, 1997). Site-specific binding may be important in anchoring the propagules at the root surface, followed by other processing, such as surface charge phenomena or hydrophobic interactions that required before colonization that continue and for growth to proceed (Recorbet and Alabouvette, 1997).

Penetration: Penetration is likely to be controlled by many different factors that include the germination of fungal spore, the structures of the plant surface, fungal compounds, and germ tube formation (Mendgen *et al.*, 1996). The means whereby wilt pathogens penetrate the roots may differ, but there are two types of penetration. Some pathogenic forms penetrate roots directly, while second pathogenic form must enter indirectly through wounds (Lucas, 1998). The most common sites of direct penetration are located at, or near the root tip of both lateral roots and tap roots (Lucas, 1998). The pathogen enters the root at the apical region where the endodermis is not fully differentiated and wilt fungi are able to grow through it and reach the developing protoxylem. *F. oxysporum* has been found to penetrate the root cap (Brandes, 1919) and hyphae forming in intercellular zone of elongation in the root of banana at 11 and 15 days after inoculations (Xiao *et al.*, 2013), while *F. oxysporum* f. sp. *dianthi* probably enters the roots through the zone of elongation in the carnation (Pennypacker and Nelson, 1972). Varieties of susceptible host muskmelon penetrated into the region of cell elongation although mechanical wounding increases infection by mechanical wounding, it is not essential for lateral root infection (Stover, 1962a).

Colonization: At the time of colonization, the intercellular mycelium advances through the root cortex until it reaches the xylem vessels and enters through the pits (Bishop and Cooper, 1983b). The fungus remains within the xylem vessels exclusively and colonize the host (Bishop and Cooper, 1983b). The colonization of the host's vascular system by the fungus is often speedy and achieved by quick formation of microconidia within the xylem vessel elements (Beckman *et al.*, 1961). The microconidia separate and are carried upward in the sap and transpiration stream (Bishop and Cooper, 1983b). The sieve plates will hinder the transport of the spores

into the leaves, and thus the spores will germinate and result in perforation of the germ tubes. Hyphae and subsequently conidiophores, then microconidia and macroconidia are formed (Beckman *et al.*, 1962).

Disease development: The most potential cause of banana wilt is a combination of pathogen activities, accumulation of fungal mycelium in the xylem and/or production of toxin, host defence responses that include production of gums, gels and tyloses, and vessel colonization of adjacent parenchyma cells (Beckman, 1987). Wilt symptoms result from severe water stress, mainly due to vessel occlusion. Many symptoms are observed, including vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission. The severely infected plants are wilted and dead, while those that are minimally affected become stunted and cannot produce fruit (MacHardy and Beckman, 1981). The vascular browning is the most common feature of internal infection (MacHardy and Beckman, 1981). Histopathological studies are useful in understanding the changes at the cellular level in response to infection (Blake, 1966; Rahe *et al.*, 1969; Clay, 1987; Pan *et al.*, 1997).

2.4.4 Pathogenicity testing

Pathogenicity is the ability of a pathogen to cause disease in which the ability represents a genetic component of the pathogen and the damage done to the host (Bos and Parlevliet, 1995). Pathogenicity is also defined as the outcome of a complex interaction in time between a host and a pathogen, each potentially variable in a changing environment to distinguish between host specificity of the pathogen and the severity of disease (Moss and Smith, 1984). The pathogens can express a wide range of virulence which refers to the degree of pathology caused by the microbes (Shaner *et al.*, 1992; Bos and Parlevliet, 1995). The extent of the virulence is usually

correlated with the ability of the pathogen to multiply within the host and may be affected by other factors such as environmental conditions.

Pathogenicity test can also be carried out to determine the host range of plant pathogenic fungi. The pathogen can differ based on the kind of plants that can be attacked. Several studies of host range have been conducted for *Fusarium* such as *F. oxysporum* from tomato (Menzies *et al.*, 1990), *F. solani* from Shisham (*Dalbergia sissoo* Roxb.) (Rajput *et al.*, 2008) and *F. equiseti* from ginseng field (Goswami *et al.*, 2008). Information from host range studies is useful in developing control strategies, especially for chemical control application.

Pathogenicity test is also used to measure or to determine virulence of different fungal isolates (Mesapogu *et al.*, 2011; Sibounnavong *et al.*, 2012). Degree of virulence can be influenced by the pathogen, host and environment. For example, the pathogen may be more or less virulent as it may be present in small or extremely large numbers or it may be in a dormant state, or it may require water. The environment may also affect the degree of virulence of the pathogen as the pathogen dispersal is influenced by wind or water (Keane and Kerr, 1997).

Fungi isolated from plants could be the pathogens that cause disease or saprophytes that can grow in the dysfunction tissues of plants and not pathogenic to healthy plants (Nelson *et al.*, 1983). Some pathogens can only cause severe diseases in plants which have been subjected to stress such as inadequate soil moisture, extreme temperature or herbicides (Burgess *et al.*, 1994).

The technique used in pathogenicity test depending on the pathogen tested. For *Fusarium* species, basically conidial suspensions with wounded methods are applied. For example, a study by Groenewald *et al.* (2006) on vascular wilt of banana caused by *F. oxysporum*, applied slightly damaged roots with conidial suspensions for

pathogenicity test. The results showed vascular wilt symptom appeared on injured inoculated plants after 6 weeks of inoculation, and disease severity caused by *Foc* isolates was based on the development of internal symptoms in the rhizomes of inoculated plants.

2.4.5 Control of Fusarium wilts

Different control methods have been attempted to inhibit the damage caused by *Foc*. However, no long-term control measures are available other than planting of resistant banana cultivars (Moore *et al.*, 1999a). Potential management of Fusarium wilt by the various methods of control were mentioned previously and some of the control strategies that have been investigated in the past. Specifically, the management practices for Fusarium wilt of banana include prevention of introducing the disease in disease-free areas, the use of disease-free tissue culture plantlets, and the use of proper sanitation methods. In the fields where *Foc* is already present, the planting of resistant cultivars is essential, if such cultivars are acceptable to the local markets (Deacon, 1984).

Studies of soils and biological controls that are suppressive naturally to Fusarium wilt disease of banana due to beneficial microorganisms have only started (Ploetz *et al.*, 2003). Many of biological control agents for Fusarium wilt diseases can be found from other crops (Alabouvette, 1999). There were discoveries of a number of synthetic chemicals that can act as strong inducers of plant defence reactions, while devoid of antifungal activity (Cohen *et al.*, 1994). Therefore, biological control and chemical activator may be favourable alternatives for managing Fusarium wilt of banana.

2.5 Biological control of Fusarium wilt disease

The application of fungicides and consumer acceptance of resistant cultivars can be very difficult, which make biological control of Fusarium wilt of banana an attractive alternative. Biological control can be realised by means of direct or indirect interaction between the control agent and the pathogen (Marois, 1990). Direct biocontrol is achieved when the control reduces the pathogen population by antagonistic effects which included different mechanisms such as competition, antibiosis, or parasitism. Competition for space, infection sites and/or nutrients, in or on roots was usually occurred (Marois, 1990). Antibiosis refers to the production of secondary metabolites by an organism that is toxic to the fungus, which may prevent or reduce germination of fungal propagules, invoke lysis, or inhibit the growth after germination, while parasitism is when a parasite attacks the mycelia and spores of the fungus in different environments (Papavizas and Lumsden, 1980). Indirect biological control works when the control agent reacts with the pathogen through the host. This interaction is also denoted as ‘cross protection’ or ‘induced resistance’, and is based on the creation of the host’s own defence system (Marois, 1990). Therefore, many biocontrol agents employ more than one mechanism to protect plants (Fravel and Engelkes, 1994).

Some microorganisms, including those of bacterial and fungal genera have been associated with biological control of Fusarium wilt diseases (Weller *et al.*, 2002). Biological control of soil-borne pathogens has been achieved by the application of several fungal species, such as the non-pathogenic isolates of *F. oxysporum* (Papavizas and Lumsden, 1980).

2.5.1 The mechanism of action of biocontrol agents

A mechanism of action described as the strategy used by the beneficial fungi against a disease-causing pathogen (Liu *et al.*, 2010). They have demonstrated the utility of several beneficial fungi such as non-pathogenic *Fusarium* isolates and *Trichoderma* spp. for biocontrol of pathogens that cause Fusarium wilt disease.

Non-pathogenic *Fusarium* isolates have been studied to control diseases such as Fusarium wilt of important agricultural crops, including banana (Gerlach *et al.*, 1999), tomato (Lemanceau and Alabouvette, 1991; Larkin and Fravel, 1998), watermelon (Larkin et *al.*, 1996; Freeman *et al.*, 2002), basil (Fravel and Larkin, 2002), celery (Schneider, 1984), chickpea (Hervás *et al.*, 1995), cucumber (Mandeel and Baker, 1991), strawberry (Tezuka and Makino, 1991), cyclamen (Minuto *et al.*, 1995), flax (Lemanceau and Alabouvette, 1991), and muskmelon (Freeman *et al.*, 2002). The non-pathogenic *F. oxysporum* had the potential to induce resistance against *Foc* race 4 for the Cavendish cultivar Williams (Gerlach *et al.*, 1999). Non-pathogenic *F. oxysporum* isolates and *Fusarium* spp. derived from symptomless banana roots provided a degree of protection against *Foc* greenhouse trial (Belgrove *et al.*, 2011).

Non-pathogenic *F. oxysporum* was reported to reduce incidence of Fusarium wilt of chickpea and its severity due to prior inoculation of seeds with the non-pathogenic isolates (Hervás *et al.*, 1995). In addition, two non-pathogenic *F. oxysporum* f.sp. *melonis* mutant strains effectively decreased the seedling mortality of both watermelon and muskmelon cultivars (Freeman *et al.*, 2002). It was also found that the non-pathogenic isolates of *F. oxysporum* isolated from tomato roots were both highly effective to control disease control against Fusarium wilt of tomato (Larkin and Fravel, 1998).