DIVERSITY OF BACTERIAL COMMUNITIES IN BACTERIAL WILT-DISEASED BANANA PLANTS

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FACULTY OF SCIENCE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2017

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THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE OF BIOLOGICAL SCIENCES

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KUALA LUMPUR

2017

ABSTRACT

The major constraint of banana production worldwide is the capacity of this crop to withstand pest and disease pressures, such as the bacterial wilt disease. In Malaysia, this disease inflicts major losses on our banana industry. Hence, initial attempt was made to detect, isolate and identify the pathogenic bacteria associated with bacterial wilt-diseased banana plants from six sampling locations around the Peninsular Malaysia. This study was further extended to identify the abundantly isolated bacterial colonies, followed by determination of the biofilm-forming ability of the identified strains. Four strong biofilm-forming strains, identified as *Enterobacter cloacae, Enterobacter hormaechei, Klebsiella pneumonia* and *Kosakonia radicincitans* were inoculated as mono- and co-cultured with the blood disease pathogenic strain, *Ralstonia syzygii* subsp. *celebesensis* into two months-old banana plants. A strong biofilm-forming strain with synergistic effects on the severity of disease symptoms was then chosen for the whole genome sequence analyses. A 16S rRNA gene sequencing metagenomics was carried out to determine bacterial communities in the symptomatic and non-symptomatic bacterial wilt-diseased banana plants. Based on the conventional method, bacteria associated with diseased banana plants were identified as *Enterobacter, Klebsiella, Pseudomonas, Kosakonia* and *Burkholderia.* Nine out of twelve isolates were identified as strong biofilm-formers. *In-vivo* assessment of mono- and co-inoculations of four strong biofilm-formers with pathogenic strain indicated that the plants co-inoculated with *K. radicincitans* and *R. syzygii* subsp. *celebesensis* were most highly susceptible to the disease. Meanwhile, plants under two treatments (co-inoculation of *R. syzygii* subsp. *celebesensis* with *E. cloacae* and *E. hormaechei,* respectively) were less susceptible. Genome sequence analyses of the *K. radicincitans* UMEnt01/12 had identified various genes related to survivability, adhesion, colonization and synergistic interactions of the bacterium. The 16SrRNA gene

sequencing metagenomics analyses further indicated that higher diversity and abundance were detected in the non-symptomatic plant as compared to the symptomatic plants. These findings suggest that the presence of biofilm-forming bacteria in diseased plants might synergistically or antagonistically affect the severity of infection caused by the pathogen. The whole genome sequence analyses had provided insights into the mechanisms of colonization, establishment and interactions with the host plant and pathogen in the vascular tissues of diseased banana plant. The diversity of bacterial populations could be associated with physiological status of the plants and this could affect the susceptibility of plants towards bacterial wilt disease. Taken together, this study implies the concepts of synergism and antagonism among the pathogen and endophytes in the diseased banana plants which may affect the occurrence and progression of disease symptoms. Although the varying abundance and diversity of the bacterial communities in both non-symptomatic and symptomatic plants could not possibly determine the contribution of the microbiota towards different plants physiological status and condition, the study had indicated comparable differences in the biodiversity of microbiota in the symptomatic and non-symptomatic bacterial wilt-diseased banana plants.

ABSTRAK

Kekangan utama pengeluaran pisang di seluruh dunia adalah keupayaan tanaman ini untuk bertahan dari tekanan perosak dan penyakit, seperti penyakit layu bakteria. Di Malaysia, penyakit ini menyebabkan kerugian yang besar kepada industri pisang kita. Oleh itu, usaha awal telah dibuat untuk mengesan, memencilkan dan mengenal pasti bakteria patogenik yang berkaitan penyakit layu bakteria tumbuhan pisang daripada enam lokasi persampelan di sekitar Semenanjung Malaysia. Kajian ini telah diperluaskan lagi untuk mengenal pasti koloni bakteria yang terbanyak dipencilkan, diikuti oleh penentuan keupayaan pembentukan-biofilem oleh '*strain*' yang telah dikenal pasti. Empat bakteria pembentuk biofilem yang kuat, dikenali sebagai *Enterobacter cloacae, Enterobacter hormaechei, Klebsiella pneumonia* dan *Kosakonia radicincitans* telah diinokulasi secara mono- dan gabungan dengan '*strain*' patogenik penyakit darah, *Ralstonia syzygii* subsp. *celebesensis* ke dalam pokok pisang berumur dua bulan. Satu '*strain*' pembentuk biofilem yang kuat dan mempunyai kesan sinergi ke atas keterukan simptom penyakit kemudian dipilih untuk analisis jujukan genom keseluruhan. Metagenomik penjujukan gen 16S rRNA telah dijalankan untuk menentukan komuniti bakteria kompleks yang mendiami pokok pisang yang dijangkiti dan tidak dijangkiti layu bakteria. Mengikut kaedah konvensional, bakteria yang berkaitan dengan pokok pisang berpenyakit telah dikenalpasti sebagai *Enterobacter, Klebsiella, Pseudomonas, Kosakonia* dan *Burkholderia.* Sembilan daripada dua belas bakteria yang dipencilkan telah dikenal pasti sebagai bakteria pembentuk biofilem yang kuat. Penilaian *in-vivo* inokulasi mono- dan gabungan empat bakteria pembentuk biofilem yang kuat bersama 'strain' patogenik menunjukkan bahawa pokok yang diinokulasi gabungan dengan *K. radicincitans* dan *R. syzygii* subsp. *celebesensis* adalah yang paling tinggi terdedah kepada penyakit. Sementara itu, pokok-pokok di bawah dua rawatan (inokulasi gabungan *R. syzygii* subsp.

celebesensis dengan *E. cloacae* dan *E. hormaechei,* masing-masing) adalah kurang terdedah (kepada penyakit). Analisis jujukan genom *K. radicincitans* UMEnt01/12 telah mengenalpasti pelbagai gen berkaitan kemandirian, pelekatan, penaklukan dan interaksi sinergi bakteria. Analisis metagenomik penjujukan gen 16S rRNA selanjutnya menunjukkan bahawa kepelbagaian yang lebih tinggi dan banyak dikesan pada pokok tanpa simptom berbanding pokok bersimptom. Dapatan kajian ini menunjukkan bahawa kehadiran bakteria yang membentuk biofilem dalam pokok berpenyakit mungkin memberi kesan sinergistik atau antagonistik terhadap keterukan jangkitan yang disebabkan oleh patogen. Analisis jujukan genom keseluruhan telah memberikan maklumat ke arah mekanisma penaklukan, pembentukan dan interaksi dengan tumbuhan perumah dan patogen dalam tisu vaskular pokok pisang berpenyakit. Kepelbagaian populasi bakteria boleh dikaitkan dengan status fisiologi tumbuhan dan ini boleh menjejaskan kecenderungan tumbuhan terdedah kepada penyakit layu bakteria. Secara keseluruhan, kajian ini menunjukkan konsep sinergi dan antagonis antara patogen dan bakteria endofitik dalam tumbuhan pisang berpenyakit yang boleh menjejaskan pembentukan dan perkembangan simptom penyakit. Walaupun pelbagai kelimpahan dan kepelbagaian komuniti bakteria di dalam kedua-dua pokok bersimptom dan tanpa simptom tidak mungkin dapat menentukan sumbangan mikrobiota ke arah fisiologi tumbuhan yang berbeza, kajian ini telah menunjukkan perbezaan biodiversiti mikrobiota yang boleh dibezakan di dalam pokok pisang berpenyakit layu bakteria yang bersimptom dan tanpa simptom.

ACKNOWLEDGEMENT

Praise be to the Almighty God, Allah SWT, for His uncountable blessings, salutations upon His Messenger, Muhammad SAW, his pure descendant, family and noble companions.

First and foremost, I would like to express my sincere appreciation to my supervisors, Prof. Dr. Thong Kwai Lin and Dr. Noni Ajam for their various contribution and suggestions in the whole process of research and finishing of the thesis. My profound gratitude to the Ministry of Higher Education, Malaysia and University of Malaya which had provided the financial support for myself throughout the years of my research.

My special thanks goes to Dr. Syarifah Aisyafaznim Sayed Abdul Rahman, Prof. Dr. Rofina Yasmin Othman, Dr. Chan Kok Gan and Dr. Rozieta Laboh for providing advice and technical help on different parts of my study. I am grateful to Cik Nur Sulastri Jaffar, Puan Junaidah and other officer and staff from the Plant and Soil Science Research Center, Malaysian Agricultural Research and Development Institute (MARDI), Serdang for their generous help and guidance through the glasshouse experiment.

I wish to thank Yap Kien Pong and Goh Share Yuan for their guidance and assistance in the whole genome and metagenomics analyses. Not forgotten are all my lab-mates in Microbiology lab, Lab 3.3, Lab A407 and all friends in University of Malaya for their support and help throughout my study.

My deepest appreciation to my mother, Sakina Md. Ibrahim, my parents-in-law, brother and sister and my whole family for their encouragement, concern and prayers. Finally, this thesis is dedicated to my beloved husband, Ronzey Mail, my son, Andhika Saifullah Ronzey, and to the memory of my father, Mohd Suhaimi Abdullah.

Without them all none of this would have been possible.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
=	Equals to
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
-VE	negative
%	Percentage
+VE	positive
±	Plus minus
$A_{260/230}$	Absorbance at 260 nm over 230 nm
$A_{260/280}$	Absorbance at 260 nm over 280 nm
bp	Base pair
cfu	Colony forming unit
CPG	Casamino peptone glucose
CTAB	Cetyl Trimethyl Ammonium Bromide
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E-value	Expectation value
g	Gram
ha	Hectare
Kb	Kilobase pair
kg	Kilogram
µg	Microgram
µl	Microliter
mg	Milligram
ml	Millilitre
mM	Milimolar
min	minute

M	Molar
ng	Nanogram
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NS	Non-symptomatic
O.D.	Optical density
O.D.c	Cut-off Optical density
PCR	Polymerase Chain Reaction
pH	Power Hydrogen
PVP-40	Polyvinylpyrrolidone 40
rpm	revolutions per minute
rRNA	Ribosomal RNA
SA	Symptomatic A
SB	Symptomatic B
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopic
sec	Second
SMSA	Semi-selective medium, South Africa
Tris-HCl	Hydrochloric tris
tRNA	Transfer RNA
TZC	Tetrazolium chloride
UV	Ultraviolet
w/v	Weight/volume
WPI	Weeks of post inoculation
v/v	Volume/volume

CHAPTER 1: INTRODUCTION

1.1 General introduction

Bananas and plantains (*Musa.* spp) are one of the most important agricultural crops in the tropics and sub-tropics with production of more than 100 million metric tons of fruits per year (Koberl et al., 2015). In Malaysia, banana plantations covered an estimated 26,000 ha of land area, which constitutes over 11.0% of the total fruit growing areas in the country ("Malaysia: Banana grower's", 2012). Bacterial wilt (BW) disease was first observed in banana plantations in Johor, Malaysia. Occurrences of the disease were recorded in the aftermath of a massive flood incident that hit the state around January 2007 (Mokhtarud-din & William, 2011; Zulperi et al., 2016). The severe outbreak of this disease had diminished enthusiasm of farmers in planting this crop since the disease is amongst the most serious fruit diseases in the country where it can spread rapidly, retards plant growth, causes critical yield losses and can rigorously impact the banana growth sector (Mokhtarud-din & William, 2011; Tengku Abdul Malik et al., 2011; Zulperi et al., 2016).

Khakvar et al. (2008) reported the presence of *R. solanacearum* in banana plants displaying symptoms of BW disease following the outbreak in 2007. Another study done by Zulperi et al. (2014a) showed that BW-diseased banana plants sampled between 2011 and 2012 were caused by *R. solanacearum* Race 2 Biovar 1. In 2013, Malaysian Agricultural Research and Development Institute (MARDI), Malaysia had identified the BDB as one of the causative agents of bacterial wilt disease of banana in Malaysia (Kogeethavani et al., 2013). This pathogen was first isolated and detected in banana plantations in Perak, Malaysia. Later on, studies and surveys by the DOA, Malaysia

indicated that *Ralstonia solanacearum* phylotype IV complex are the causative agents of BW disease of banana plant in Malaysia (Timin et al., 2014). On the other hand, there were also reports on endophytic bacterial species which are associated with the wilting or rotting diseases in many plants (Masyahit et al., 2009; Nishijima et al., 2004; Promsai et al., 2012; Wang et al., 2010; Zhang & Nan, 2013). Under natural conditions, *Enterobacter* spp. are ubiquitous endophytic bacteria (Nie et al., 2002; Taghavi et al., 2010) but could turned pathogenic when fluctuations in the environment favour their rapid growth (Wang et al., 2010).

In recent years, the study of multispecies synergistic interactions involved in the expression of disease symptoms is emerging as a new important subject for better understanding of microbial diseases and this fact should be considered in disease control (Rossmann et al., 2012; Short et al., 2014). Furthermore, BW disease of banana in Malaysia could be caused by an amalgamation of a wide range of pathogenic and endophytic microbial interaction. Hence, this whole study was conducted in order to identify not only the pathogenic agents of bacterial wilt-diseased banana, but also diverse culturable and non-culturable bacterial communities in the symptomatic and non-symptomatic plants.

The whole genome annotations and analyses of a biofilm forming bacterial strain were completed in order to assist in the understanding of synergistic ability of this bacterium in enhancing the BW-diseased development and progression. Furthermore, the application of techniques based on analysis of nucleic acids (DNA or RNA) directly extracted from environmental samples is essential in microbial diversity studies. They can provide information in a culture-independent way, and exclude the limitations and bias from the low cultivable portion of bacteria in these communities (Andreote et al., 2009). It has been reported that a 16SrRNA metagenomics offered a glimpse into the phylogenetic diversity of cultured and uncultured microorganisms (Tringe et. al., 2005).

Thus, the 16S rRNA metagenomics approach was explored in order to provide an overview of the complex interactions of various potential pathogenic and beneficial plant-associated bacteria in BW-diseased banana plants.

1.2 Objectives of the study

General objective:

To elucidate the diversity of culturable and non-culturable bacterial communities in the symptomatic and non-symptomatic bacterial wilt-diseased banana plants.

Specific objectives of the study are as follows:

1. To isolate the pathogenic agent of bacterial wilt disease of banana from the selected orchards in Peninsular Malaysia.
2. To identify the abundantly isolated bacterial colonies from the symptomatic and non-symptomatic bacterial wilt-diseased banana plants and to determine the ability of the identified strains to form biofilm.
3. To determine the effects of mono and co-culture inoculation of the strong biofilm-forming bacterial strains with a blood disease pathogen in glasshouse conditions.
4. To analyse the whole genome sequence of *Kosakonia radicincitans*, a strong biofilm-forming bacterial strain.
5. To decipher and compare the diversity and abundance of bacterial communities in the symptomatic and non-symptomatic bacterial wilt-diseased banana (*Musa paradisiaca* cultivar Nipah) plants by employing a 16S rRNA metagenomics approach.

CHAPTER 2: LITERATURE REVIEW

2.1 The bananas and plantains (*Musa* spp.)

Bananas and plantains which belong to the Genus *Musa* (Family *Musaceae*; Order Zingiberales), are monocotyledonous flowering plants. Each plant producing a single false stem (pseudostem) consisting of leaves sheaths and an underground true stem (corm) that is able to produce suckers which is crucial for vegetative reproduction. Banana suckers continue to emerge from a single mat year after year, making it a perennial crop (Food and Agriculture Organization, 2003). The vast majority of banana cultivars involved the inter- and intraspecific crosses between two diploid wild species, *Musa acuminata* and *Musa balbisiana* (D'Hont et al., 2012; Heslop-Harrison & Schwarzacher, 2007). The *Musa* crop is distributed mainly on the margins of tropical rainforests (Wong et al., 2002). The centre of origin of this fruit crop is in South-East Asia, extending from India to Polynesia (Daniells et al., 2001; Heslop-Harrison & Schwarzacher, 2007).

Banana is a rich source of carbohydrate and some minerals, mainly phosphorus, calcium and potassium. It is also rich in vitamin C and contains significant amounts of several other vitamins (Siddique, 2014). Moreover, it has great medicinal value (Das et al., 2009; Kumar & Bhowmik, 2012). These *Musa* spp. are generally divided into two types based on the fruits produced. The sweeter forms that are eaten raw are known as bananas while plantains (cooking banana) are starchier and lower in sugar, peeled with knife when unripe and then cooked (Heslop-Harrison & Schwarzacher, 2007). This cooking-typed banana will still be green in colour when they are ripe and hardly eaten raw because of the high starch content. In general, about half of banana families in the world are eaten raw while the other half are cooked as vegetables (Simmonds, 1966).

Globally, over 100 million metric tons of fruits are produced annually (Koberl et al., 2015). The 2013 – 2014 statistics showed that India led the world in banana production, while among the Asian countries, Philippines is the largest exporter in the region with 2.7 million tonnes exports per year (Food and Agriculture Organization of the United Nations, October 2015).

2.1.1 Bananas in Malaysia

Banana is listed among the ten most popular economic crops and the second most commonly grown fruit crop (after durian) in Malaysia (Mak et al., 2001; Nik Hassan, 2003). This fruit crop covers about 26,000 ha with a total production of 530,000 metric tonnes ("Banana – The Challenge", 2015). Johor, Pahang and Sarawak are the three major banana producing-states in this country (Mokhtarud-din & William, 2011). About 50% of the banana growing land is cultivated with Pisang Berangan or the Cavendish type, and the remaining popular cultivars are Pisang Mas, Pisang Rastali, Pisang Raja, Pisang Awak, Pisang Abu, Pisang Nangka and Pisang Tanduk ("Malaysia: Banana grower's", 2012). In Malaysia, bananas are cultivated mainly as a cash crop and this giant herb can also be found temporarily intercropped with oil palms, rubber trees or other perennial crops (Mak et al., 2001; Nik Hassan, 2003). Most of the bananas produced were consumed locally and only 10 – 12% of the total banana production is exported, mainly to Singapore, Brunei, Hong Kong and the Middle East ("Banana – The Challenge", 2015).

2.1.2 Bananas pest and diseases in Malaysia

Globally, bananas and plantains are subjected to wide range of pest and devastating diseases (Sequeira, 1998), and there are continuous challenges to existing varieties by new emerging diseases and newly virulent strains (Heslop-Harrison & Schwarzacher, 2007). The severity and occurrence of the outbreaks and plant damage depend on three inter-related factors namely environmental conditions, specific banana variety and specific pest or disease (Nelson et al., 2006).

Major banana diseases reported in Malaysia are caused by fungi and bacteria. Notably, Fusarium wilt (causal agent: *Fusarium oxysporium* f. sp. *cubense*) and bacterial wilt disease (causal agent: *Ralstonia* species-complex), pose a serious threat to this crop plant (Mokhtarud-din & William, 2011). These wilting banana diseases had severely impact the banana growth sector, widespread rapidly and retard banana plant growth resulting in critical yield losses. (Nik Hassan, 2003; Zulperi et al., 2016). Meanwhile, some of the common pest of banana in this country are *Cosmopolites sordidus* (Banana root borer), *Odoiporus longicollis* (Banana stem weevil), *Erionota thrax* (Banana skipper), *Chaetanaphotrips signipennis* (thrip), *Rodophilus similis* (burrowing nematode) and *Bactrocera papayae* and *B. carambolae* (fruit fly) (Mokhtarud-din & William, 2011).

2.2 Bacterial wilt disease of banana

Bacterial wilt disease was first described by Erwin F. Smith in potato, tomato and eggplant in 1896, and subsequently in tobacco in 1908 (Álvarez et al., 2010). In general, bacterial wilt in plant is considered as the single most destructive bacterial disease due to its extreme aggressiveness, wide geographic distribution, and unusual broad host range (Meng, 2013; Prior et al., 2013).

The bacterial wilt (BW) or vascular wilt of banana is known as Moko, Bugtok and Blood disease. Although these three are closely related, the distribution of each disease is quite distinct (Fegan, 2005). The causative agents are members of the *Ralstonia* species complex. Since long ago, BW disease constitutes a great threat to the banana industries in this world, mainly in poor countries because 1) among the wild diploids, there are very few sources of resistance to BW disease, while triploid banana and plantain varieties are all susceptible; 2) dissemination of disease through vectors are rapid and unstoppable; and 3) control of the disease are expensive and can be implemented only by large-scale producers (Sequeira, 1998). Hence, this disease inflicted major losses to the small growers of banana and plantains worldwide.

Symptoms of the disease varies depending on the strain which cause the infection, mode of transmission of the pathogenic strain, the age of the host plant and the reaction of the host plant itself (Fegan, 2005). Infection via wounds of the roots and rhizome produces symptoms of yellowing, stunting and wilting of the foliage, vascular discoloration and reddish-brown fruit rot (Álvarez et al., 2010; Remenant et al., 2011). Some matured plants might show no apparent symptoms but the fruit pulp will rot and the plants will eventually die (Albuquerque et al., 2014; Fegan & Prior, 2006). In contrast, insect transmitted Moko produces symptoms of shrivelling and blackening in the male buds and peduncle with further discolouration of vascular tissues as the infection becomes systemic and moves through the entire plant (Fegan, 2005).

In the 1890s, Moko disease was first identified in Trinidad when it caused severe losses of ABB genotype banana (Bluggoe subgroup) (Fegan, 2005). This disease which infect triploid banana, heliconia (*Heliconia* spp.), and other ornamental *Musaceae* plants is known to be caused by *Ralstonia solanacearum* strains (Albuquerque et al., 2014). All of these Moko disease-causing strains are phylogenetically distributed within phylotype II (American origin) and further subdivided into lineages IIA and IIB (Albuquerque et al.,

2014). This disease affects the smallholder farmers in many parts of Latin America and is also present in the Caribbean. It is officially recorded in Belize, Brazil, Colombia, Costa Rica, Ecuador, El Salvadore, Grenada, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Peru, Surinam, Trinidad and Venezuela (Fegan, 2005).

Bugtok or tapurok disease has only been reported in Philippines and first observed as early as 1950s in Cardaba and Saba varieties of bananas (Fegan & Prior, 2006). Although the disease is caused by the same pathogen as Moko, it differs in symptoms, which is the result of the host reaction by these varieties which are of the Balbisiana genotype (Fegan, 2005). Bugtok is incompletely systemic where the symptoms are confined to the floral raceme and the normal route of infection is via the inflorescence. The infection caused red or brown discolouration of fruit pulp but vascular discolouration is generally not found beyond the peduncle except in severe cases (Eden-Green et al., 1994; Fegan, 2005; Thwaites et al., 2000). Unlike Moko, the diseased plants do not wilt and suckers are also generally not affected (Fegan, 2005).

Blood disease was first reported in 1906 in the Saleiren Islands off the coast of Sulawesi (formerly Celebes) when it caused the abandonment of newly-established dessert banana plantations (Hayward, 1992). Quarantine restrictions which prevented the exportation of bananas from that region appeared to control the spread of the disease until another incidence was reported in West Java in 1987 (Eden-Green & Sastraatmadja, 1990). Since then, the disease had spread to Lombok Island and West Nusa Tenggara (Allen et al., 2005), followed by detection in Sumatra, West Kalimantan the Moluccan Islands and Irian Jaya (Baharuddin, 1994). Blood disease is estimated to be spreading at the average speed of 100 km/year and with some areas that reached up to 200 km/year (Habazar et al., 2012). This banana disease is soil-borne and may also originate from contaminated soil or water, but epidemics due to nonspecific mechanical transmission by insects visiting banana flowers and human activities (Hermanto & Emilda, 2013; Subandiyah et

al., 2005). According to previous reports, the prevalence of blood disease is limited only in Indonesia (Fegan & Prior, 2006). Until recently, the bacterial wilt disease of banana in Peninsular Malaysia was identified to be as similar as the blood disease (Kogeethavani et al., 2013; Teng et al., 2016).

Pathogenic strains from Moko and Bugtok disease were pathogenic to banana and all solanaceous hosts (tomato, eggplant and tobacco), while blood disease strains produced symptoms only in banana.

2.3 The causative agents of bacterial wilt disease of banana

Globally, bacterial wilt disease of banana generally known as Moko, Bugtok and Blood disease are caused by *R. solanacearum* species complex (Fegan & Prior, 2006). *R. solanacearum* strain causing Moko and Bugtok belongs to phylotype II while Blood disease bacterium (BDB), known as *Ralstonia syzygii* subsp. *celebensis* (Safni et al., 2014), which cause the Blood disease belongs to phylotype IV. Within the phylotype IV, there is a cluster of closely related strains consisting of *R. solanacearum, Ralstonia syzygii* (causative agent of Sumatra disease of cloves) and BDB (Remenant et al., 2011).

2.3.1 *Ralstonia solanacearum* species-complex

Ralstonia solanacearum Yabuuchi et al. (1995) is a soil-borne vascular pathogen which belongs to the family *Ralstoniaceae* in β-Proteobacteria group (Stackebrandt et al., 1988). It is a bacterium with wide geographic distribution which is responsible for bacterial wilt on more than 200 plant species from 50-53 different botanical families, including important crops such as potato, tomato, eggplant, pepper, tobacco, ginger, peanut and

banana (Elphinstone et al., 2005; Kubota et al., 2008; Meng, 2013). The most important widespread hosts belong to the *Solanaceae* and *Musaceae* families (Álvarez et al., 2010).

R. solanacearum has been considered as a heterogeneous species or a "species-complex" (Fegan & Prior, 2005), defined as a cluster of closely related strains with high genetic variation within the species due to long evolution occurring independently in various areas on different hosts (Buddenhagen & Kelman, 1964; Gillings & Fahy, 1994). Based on the 16S rDNA sequence analysis, Taghavi et al. (1996) expanded the concept of the *R. solanacearum* species complex by including two closely related species from Indonesia, *Ralstonia syzygii* subsp. *syzygii* (causative agent of Sumatra disease of clove trees) and *Ralstonia syzygii* subsp. *celebesensis* (BDB), the agent of banana blood disease.

Bacterial wilt caused by *R. solanacearum* race 2, biovar 1, known as Moko disease can infect triploid banana, *Heliconia* spp., and other ornamental *Musaceae* plants (Albuquerque et al., 2014; Cook et al., 1994). The new hierarchical classification scheme by Fegan and Prior (2005) had revealed a subdivision of this species into four phylotypes, which were correlated with the geographical origins of the strains, consisted of phylotype I which include strains from Asia, phylotype II, strains from America, phylotype III, strains from Africa and phylotype IV, strains from Indonesia, Australia and Japan (including the close relative strains, *Ralstonia syzygii*). These phylotypes were then subdivided into 52 different groups of strains, named sequevars, according to the endoglucanase (*egl*) nucleotide sequence (Peeters et al., 2013) (Figure 2.1).

This new classification scheme has been employed by a large number of researchers to characterize *R. solanacearum* species complex populations from different parts of the world (Albuquerque et al., 2014; Fonseca et al., 2014; Lin et al., 2014; N'Guessan et al., 2012; Ramsubhag et al., 2012; Sagar et al., 2014; Xue et al., 2012). This phylotyping scheme had also categorized the Moko pathogen into four distinct sequevars: IIA-6, IIA-

24, IIB-3, and IIB-4 (Albuquerque et al., 2014; Cellier & Prior, 2010; Fegan & Prior, 2005).

2.3.2 Blood Disease Bacterium (BDB)

The pathogenic bacterium of banana blood disease, the blood disease bacterium (BDB) was first isolated and named *Pseudomonas celebensis* by Gaumann in 1920 (Machmud 1985), but the original culture deposited as a type strain no longer exist, hence the name is taxonomically invalid (Remenant et al., 2011). Since then, the pathogenic agent of blood disease is known as BDB, until the polyphasic taxonomic revision by Safni et al. (2014), reclassified this bacterium as *R. syzygii* subsp. *celebesensis.*

On the Tetrazolium chloride (TZC) medium, the phenotypic characteristics of BDB is different from *R. solanacearum.* BDB is a slow-growing bacterium which produced small, non-fluidal or slightly viscid colonies with red centers and well-defined white margins on the TZC agar (Hayward, 1992; Supriadi, 2003). The isolates are oxidase positive, arginine dihydrolase negative and, unlike most of *R. solanacearum* strains (including those causing moko disease), BDB do not reduce nitrate or denitrify. BDB is not pathogenic to *Heliconia* spp. in the wild and the artificial inoculation showed that the bacterial strain produced blood disease symptoms only in banana and not to other Solanaceous hosts (Cellier & Prior, 2010).

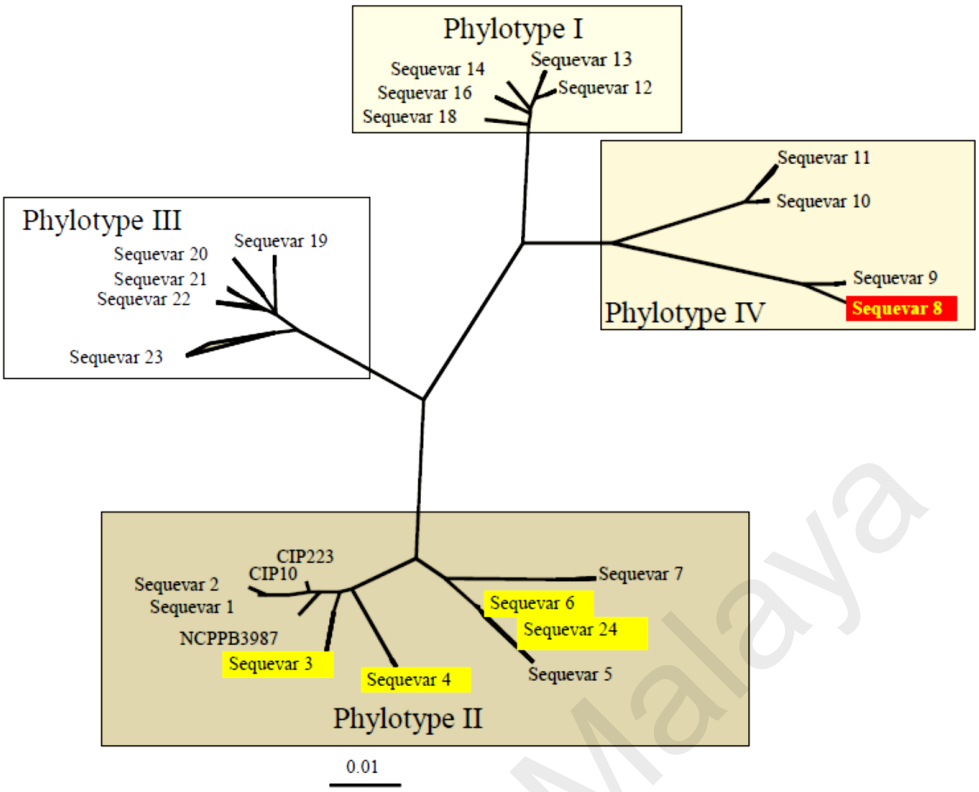

Figure 2.1: Phylogenetic tree generated from partial endoglucanase gene sequence data showing the phylogenetic relationships of sequevars and phylotypes. The bar indicates 1 nucleotide change per 100 nucleotide positions. Sequevars highlighted in yellow indicate those sequevars containing moko disease causing strains and the sequevar highlighted in red contains blood disease causing strains. Source of photo: Fegan (2005)

2.4 Epidemiology of bacterial wilt disease of banana in Malaysia

In Malaysia, the first outbreak of banana wilt was observed in banana plantation in Muar, Johor, around the early 2007 (Mokhtarud-din & William, 2011; Zulperi et al., 2014b). This BW disease heavily attacks the cooking varieties *Pisang Abu*, *Pisang Nipah* and *Pisang Raja* with up to 70 – 100% infection levels. Among the dessert varieties, *Pisang Rastali* and *Pisang Berangan* reached infection level of up to 64% (BAPNET Bulletin). Disease status survey in that year showed that 60.7% of 3,212 ha banana orchards in Johor were found to be infected by banana bacteria wilt (Timin et al., 2014).

Within four years, the disease had spread to other states of Kedah, Perak, Kelantan, Negeri Sembilan, Malacca, and Pahang (BAPNET Bulletin). Denny (2007) stated that the tropical and subtropical condition with temperate climate like Malaysia is conducive for the development of disease in the infected region. Several physical symptoms of BW disease are yellowing and wilting of the oldest leaves, which become necrotic and led to collapse of the leaves. The symptoms then spread to the younger leaves, which developed pale green panels before becoming necrotic (Zulperi et al., 2014a). The disease also caused blackened, stunted or twisted in young suckers and tendency for the whole plant to collapse. In older plants, the most frequent symptoms are wilting and yellowing of the foliage and appearance of dark brown stripes corresponding to the infected vascular bundles beneath the epidermis. In matured plants, fruits appeared normal and unaffected but the internal of banana fruits displayed brownish black discolouration and eventually rotted (Figure 2.2). (Fegan & Prior, 2006).

Figure 2.2: Some of the physical symptoms shown by bacterial wilt-diseased banana plants in Malaysia

The symptoms observed on banana plants in Malaysia are very similar to Moko disease, which originated in Central America and were described in many other countries, where the causative microorganism was identified as *Ralstonia solanacearum,* phylotype II (Fegan & Prior, 2006; Ilagan et al., 2010; Perez et al., 2008). However, similar symptoms are also observed in the blood disease of bananas and plantains in Indonesia (Allen et al., 2005; Fegan, 2005; Remenant et al., 2011), induced by Blood Disease Bacterium (BDB), known as *Ralstonia syzygii* subsp. *celebesensis* (Safni et al., 2014).

In the early years of BW infection in Malaysia, Khakvar et al. (2008) had reported the presence of *R. solanacearum* in the Moko diseased banana plant samples collected from banana plantations in several regions around the Peninsular Malaysia. In 2015, the isolation and characterization of *R. solanacearum* race 2 biovar 1 from Moko diseased banana plants in Malaysia was announced by Zulperi and Sijam (2015). Various sample materials were collected from banana plants of cultivar *Musa paradisiaca* cv. Horn and *M. paradisiaca* cv. Nipah within 2011 to 2012 (Zulperi & Sijam, 2015; Zulperi et al., 2014a). Later on, Zulperi et al. (2016) further reported that the phylogenetic analysis of the isolated *R. solanacearum* strains based on the phylotype and sequevar scheme had classified the pathogenic bacterium as *R. solanacearum* phylotype II sequevar 4 (II/4). Meanwhile, Malaysian Agricultural Research and Development Institute (MARDI) had identified BDB as the causative agent of BW disease of banana in Malaysia (known as blood disease), which was first detected at Perak, Malaysia in 2013 (Kogeethavani et al., 2013). Recently, Teng et al. (2016) has reported another finding of blood disease occurrence caused by BDB in banana plantations in Selangor, Malaysia.

Based on the surveys and studies done by the Department of Agriculture (DOA), Malaysia (Timin et al., 2014), the researchers had concluded that banana samples from Peninsular Malaysia and Sabah were found to be infected by *R. solanacearum* phylotype IV, which was reclassified as *Ralstonia syzygii* subsp. *indonesiensis* (Safni et al., 2014)

and also BDB. Hence, BW disease of banana in Malaysia could be either Moko disease, blood disease or both which caused by members of *R. solanacearum* species-complex, including the BDB (*R. syzygii* subsp. *celebesensis*).

2.5 Isolation and identification of the pathogenic agents of bacterial wilt disease of banana

2.5.1 Cultural and serological techniques

Isolation of the pathogenic agents of bacterial wilt disease from symptomatic plant samples can easily be performed by using non-selective, casamino peptone glucose (CPG) or Kelman's Tetrazolium-chloride (TZC) media (Chaudhry & Rashid, 2011) due to the high density of the pathogen in the tissues. Non-symptomatic plants caused more difficulties due to low number of viable cells. The most problematic isolation is from the soil due to the presence of other microorganisms, many of which are fast-growing bacteria (Denny & Hayward, 2001). Several semi-selective media have been described for the isolation of *R. solanacearum* (Aley & Elphinstone, 1995; Elphinstone et al., 1996; Ito et al., 1998). However, none is highly satisfactory for selection of *R. solanacearum* because the media are unable to suppress growth of related and/or unrelated Gram-negative bacteria (Denny & Hayward, 2001). Nevertheless, a modified semi-selective medium, South Africa (SMSA) has been used successfully in Europe for the isolation from latently infected material or difficult substrates like soil, waste, or surface water (Elphinstone et al., 1996; Wenneker et al., 1999).

Meanwhile, detection and identification of the phytopathogens using serological agglutination tests allow a rapid disease diagnosis in the field (Danks & Barker, 2000). Methods like enzyme-linked immunosorbent assays (ELISA) and field kit in the form of

a lateral flow device which utilize specific monoclonal and polyclonal antibodies are now and accepted methods for screening of plant samples for latent infections (Danks & Barker, 2000; Denny & Hayward, 2001).

2.5.2 Molecular techniques

Using the molecular technique, several primers have been developed for the detection of *R. solanacearum* strains using polymerase chain reaction (PCR) assays (Villa et al., 2003). There are significant progress in detection and identification of *R. solanacearum* species-complex from different samples such as plants and soil by using PCR technique. Seal et al. (1993) have successfully developed a primer pair, OLI1/Y2 (which produced approximately 288-bp bands) and Opina et al. (1997) with primer pair, 759/760 which consistently amplified a 282-bp fragment from all *R. solanacearum* strains, and DNA of the closely related bacteria, *Ralstonia syzygii* (Sumatra disease of cloves) and the blood disease bacterium (BDB) affecting bananas. Two pairs of primers, PS96-H/PS96-I permitted specific amplification of only *R. solanacearum* strains (Hartung et al., 1998; Seal et al., 1992). Table 2.1 summarized the details of primers useful for the detection and identification of *R. solanacearum* and closely related species.

Table 2.1: PCR primers useful for *R. solanacearum* and closely related species.

Primer	Primer sequence (5' to 3')	No. of bases amplified	Specificity*	References
Y2 OLI1	CCCACTGCTGCCTCCCGTAGGAGT GGGGGTAGCTTGCTACCTGCC	287-288	*Rs* + related	Seal et al., 1993
759 760	GTCGCCGTCAACTCACTTTCC GTCGCCGTCAGCAATGCGGAATCG	281	*Rs* + related	Ito et al., 1998 Opina et al., 1997
PS96-H PS96-I	TCACCGAAGCCGAATCCGCGTCCATCAC AAGGTGTCGTCCAGCTCGAACCCGCC	148	*Rs*	Hartung et al., 1998 Seal et al., 1992
pehA#3 pehA#6	CAGCAGAACCCGCGCCTGATCCAG ATCGGACTTGATGCGCAGGCCGTT	504	*Rs*	Gillings et al., 1993

*Primer pairs OLI1/Y2 and 759/760 amplify fragments of *Ralstonia solanacearum* (*Rs*), *Ralstonia syzygii*, and the blood disease bacterium (related), but not from *R. pickettii*, *R. eutropha* or other bacteria. Primer pair PS96-H/I amplifies DNA fragments of *R. solanacearum.* Primer pair pehA#3/#6 amplifies DNA fragments of *R. solanacearum*, while amplification from *Ralstonia syzygii* and the blood disease bacterium were not tested

2.6 The plant microbiome

Current advances in agrobiology research is gaining insight into the composition of phytobiome with the aim of improving crop quality and health. Study of phytobiome include the entire system of factors that affect or affected by the plants or the plant environment (Leach, 2015). Three elements in the disease triangle constituted the interactions of a susceptible host, virulent pathogens and favourable environment (Scholthof, 2007). Among the environmental factors, the functioning plant microbiome may significantly contribute to the disease incidence and severity, and also determine plant health and productivity (Berendsen et al., 2012). These plant microbiome might interact with their host plants through pathogenesis, symbiosis or commensal relationships (Ramey et al., 2004).

2.6.1 Endophytic bacteria

Plants are complex micro-ecosystems with different habitats which are exploited by a wide variety of bacteria. Endophytes are the microorganisms residing within plant tissues (the endosphere), such as leaves, roots or stems (Turner et al., 2013). These endophytes present in various plants, often without any apparent adverse effects on the host system (Reinhold-Hurek & Hurek, 2011; Ting et al., 2008). However, Mano et al. (2007) stated that the endophytic communities can also be pathogenic or non-pathogenic to their respective host plants.

Endophytic bacteria might also act as antagonists in controlling phytopathogens attack through several mechanisms such as competition for ecological niches or substrates, production of inhibitory allelochemicals, and induction of systemic resistance in host plants to a broad spectrum of pathogens (Compant et al., 2005). Barka et al. (2002)

elucidated that a plant growth-promoting rhizobacterium (PGPR), *Pseudomonas* sp. inhibits the growth of *Botrytis cinerea* through the disruption of cellular membranes which induce cell death of the pathogen.

2.6.2 Biofilm-forming endophytic bacteria

Biofilm-type structure that vary from small clusters of cells to extensive biofilms were developed in those associations (Ramey et al., 2004). Bacterial biofilm consist of microbial aggregates which forms by bacterial adhesion, growth and expansion process (Rafique et al., 2015). Bacterial cells in this complex biofilm network act less as individual entities and more as a collective living system (Kumar et al., 2016). The biofilms are typically made of water and the bacterial cells, enclosed in a hydrated matrix of proteins, nucleic acids, and polysaccharides produced by the microbes (Kumar et al., 2016; Sutherland, 2001), which provide physical barriers against the diffusion of antibiotics, defence substances, or other important compounds from the host and also protect against hostile environmental conditions such as UV radiation, pH changes, osmotic stress, and desiccation (Bogino et al., 2013; Gilbert et al., 1997). Biofilm formation is predominant property in bacterial life style with great importance in exhibiting both beneficial and detrimental activities in their host (Rafique et al., 2015). Certain bacteria in biofilm matrices are symbiotically associated with their host plant to induce plant growth and to protect against phytopathogens (through a process termed biocontrol), while others are involved in pathogenesis (Bogino et al., 2013). For instance, formation of biofilm by the vascular wilt pathogens contribute to the virulence of these bacteria through various mechanisms such as blockage of xylem vessels, increased resistance to plant antimicrobial compounds, and enhanced colonization of specific site in plant tissues (Mansfield et al., 2012). Muthoni et al. (2012) reported that *R.*

solanacearum cause plant wilting due to formation of slime that surrounds the bacterial mass in the stem vascular bundles which blocked the water transport system. Jacob and Kim (2010) showed that a biofilm-forming bacterium, *Enterobacter* sp. can cause vascular blockage and early withering in cut flowers.

Meanwhile, PGPR such as *Pseudomonas* sp. and *Azospirillum* sp. form biofilms and enhance root growth and provides opportunities for the plant to acquire maximum nutrients for the better growth (Rafique et al., 2015). A study of plant growth-promoting bacteria (PGPB) and nitrogen-fixing endophytes revealed that these bacteria colonized the plant and also produced biofilm (Meneses et al., 2011).

In general, as a first step in the colonization process, extracellular polymeric substance (EPS), flagella, pili, bacterial exudates and signalling molecules have been shown to be crucial for the biofilm formation (Meneses et al., 2011; Rodríguez-Navarro et al., 2007). This EPS production is essential in the anchoring phase to initiate and maintain contact between cells, where the bacteria become irreversibly bound to the tissue surface (Bogino et al., 2013).

2.6.3 Synergistic and antagonistic interactions of plant microbiome

Current concept in microbial plant diseases suggested that synergistic interactions of microbiomes in plant can lead to disease complexes (polymicrobial diseases) where diverse saprophytic or opportunistic microorganisms are involved in the expression of symptoms of these plant diseases (Berg, 2009; Lamichhane & Venturi, 2015). A review by Lamichhane and Venturi (2015) had discussed evidences of synergistic interactions among the microbial communities in plants which may increase disease severity as compared to a single pathogen infection. The exploration of synergistic commensal–

pathogen and pathogen–pathogen interactions which led to increase in diseased severity had emerge as remarkable findings in human pathology studies (Bosch et al., 2013; Peters et al., 2012). In many earlier plant pathology researches, the concept of monospecies/monostrain infection was commonly reported (Lamichhane & Venturi, 2015). However Fitt et al. (2006) had stated that, in plants, co-infection by more than one pathogen are more frequently reported as compared to the infection in animals.

Due to the phytopathogen infections, systemic acquired resistance of plant might be induced (Rojas et al., 2014), which resulted in an alteration of the physiological status within the plant that leads to the arrival and colonization by other microbes (Melcher et al., 2014). A few investigations on plant-microbe interactions often reveal the presence of multiple microbes in endophytic association with plants (Ding et al., 2013; Melcher et al., 2014). These endophytic bacteria can also antagonize pathogens through several strategies such as, competition for colonization sites by producing allelochemicals (siderophores, antibiotics, biocidal, lytic enzymes, and detoxification enzymes), degradation of pathogen's virulence factors or the interference with pathogen's quorum sensing (Bulgari et al., 2014). Several endophytic microbes can also be biocontrol agents in controlling the proliferation of other microorganisms in plant (Santoyo et al., 2012). Hence, evaluation needs to be focused on all the microorganisms isolated from the infected plant, as multispecies interactions and consortia might be involved in establishment and progression of the disease (Lamichhane & Venturi, 2015).

2.7 Whole genome sequence of plant-associated bacteria

The bacterial family Enterobacteriaceae consists of many plant-associated bacteria, including the phytopathogens and plant growth promoting bacteria (PGPB). More genome sequences have been determined from this family than from any other The whole

genome sequence (WGS) analyses are widely used platforms to provide new insights into the genetic characteristics of many plant pathogenic bacteria and PGPB from this family such as, *Enterobacter, Klebsiella, Erwinia* and *Pseudomonas* (Bell et al., 2004; Fouts et al., 2008; Joardar et al., 2005; Sebaihia et al., 2010; Taghavi et al., 2010; Witzel et al., 2012).

For instance, the analysis conducted on the genome sequences of *Enterobacter* sp. 638 has revealed key functions in plant growth promotion and plant protection, as well as the description of genes related to endophytic colonization, establishment and interaction with the host plant (Taghavi et al., 2010). Similarly, this WGS platform had revealed the ability of a diazotrophic endophyte, *Klebsiella pneumoniae* 342 in adopting efficient endophytic lifestyle through antibiotic resistance mechanisms, surface attachments, secretion systems, insertion element and transporter contents (Fouts et al., 2008). Meanwhile, the exploration of genome sequences of phytopathogenic bacterium, *Erwinia carotovora* subsp. *atroseptica* had produced a wealth of information on novel and shared genes which are potentially involved in pathogenicity and various metabolisms (Bell et al., 2004).

2.8 The culture-independent techniques in bacterial identification

Earlier understanding indicated that the endophytic bacteria were present in low abundance as compared to the rhizoplane colonizers (Compant et al., 2010; Hallmann, 2001). However, studies on the microscopic examination of tissue homogenate have revealed substantial numbers of these microorganisms and the non-cultivability is the prime reason for them escaping the conventional identification techniques (Thomas & Reddy, 2013; Thomas & Sekhar, 2014; Thomas & Soly, 2009). Hence, investigators want

to consider a culture independent approach to detect all microbial components of the phytobiome in their analyses (Melcher et al., 2014).

2.8.1 Application of metagenomics analyses in plant microbiome identification

In recent decades, study of microbiomes structure mainly by amplicon sequencing and microscopy have been gaining interest of many researchers (Berg, 2015). The investigations of plant-associated bacteria often reveal the presence of multiple microorganisms and the interaction among these phytobiomes microbes have consequences for the host plant (Melcher et al., 2014). Phytobiomes studies provide precise insights into the mechanisms and consequences of the occurrence and development of plant disease and resistance, also help to identify microbial indicators which associate with the progression of disease and resistance (Leach, 2015). For instance, studies showed that presence or absence of the pathogenic microbe can greatly influence microbial community composition more than the environmental factors (Bulgari et al., 2011) and the genotype of the host plants (Reiter et al., 2002). Another study indicated that rhizosphere communities on the infected orange trees (citrus greening disease) were different from those on the uninfected trees which elucidated that the disease is associated with detectable shifts in the composition and functional of the microbiomes in the plant (Trivedi et al., 2012). In addition, the exploration of fluctuations in the diversities of marine microbes has revealed the responses of such ecosystem to climate change and anthropogenic pollution (Coelho et al., 2013).

Microorganisms that live in association with plant tissues (known as plant microbiota) are less investigated for metagenomics compared to soils and marine microbial (Wang et al., 2008). Metagenomics is the culture independent analyses (without requirement for laboratory culture) of an assemblage of microbial genomes (termed the metagenome)

(Schloss & Handelsman, 2005) which use an approach of high-throughput DNA sequencing technology to access the uncultured majority (Morgan et al., 2010). The taxonomic content of the samples could be analysed through a random shotgun sequencing of the extracted DNA from the sample or to use a targeted approach in which only one particular gene is amplified and sequenced (Mitra et al., 2011). The taxonomic classification of sequence reads is crucial in the circumstances where the etiologic agent is unknown or the symptoms could be produced by multiple species of pathogenic microbes (Bernardo et al., 2013; Melcher et al., 2014).

Metagenomics analysis was first studied using 16S rDNA clone libraries and automated Sanger sequencing which produced long, high quality reads (generally 700 – 900 bp), yet limited to certain number of samples that could be sequenced in a single run and by cost (Goldberg et al., 2006). The invention and emergence of next generation sequencing (NGS) technologies has greatly benefited metagenomics by allowing for large amounts of data to be produced at a low cost (Metzker, 2010). These technologies had changed our genome sequencing approaches and the associated timelines and costs, also accelerated and altered a wide variety of types of biological inquiry that have historically used a sequencing-based readout **(**Mardis, 2008**)**.

The pyrosequencing and metagenome-based studies have shown an extensive diversities of unculturable endophytic communities occupying various niches with substantial insights into their lifestyles and metabolic capabilities (Bulgarelli et al., 2012; Sessitsch et al., 2012; Tringe et al., 2005; Wang et al., 2008).

2.8.2 16S rRNA-based metagenomics analyses

The ribosomal RNA (rRNA) genes (16S or 18S rRNA) that are present in all living cells are widely used for the phylogenetic studies and as the target of amplicon sequencing (Pace et al., 1985). The 16S rRNA gene is highly conserved in prokaryotes and yet hypervariable between species, which is a good candidate marker for the taxonomic analyses of bacteria by using various sequencing technologies such as 454 pyrosequencing, Illumina, Solid and Ion Torrent (Devine et al., 2012; Oulas et al., 2015).

The nine hypervariable regions (V1-V9) of 16S rRNA genes demonstrate considerable and differential sequence diversity among different bacteria but, no single hypervariable region is able to distinguish among all the bacteria (Shah et al., 2011). Nevertheless, hypervariable regions V2 (nucleotides 137-242), V3 (nucleotides 433-497) and V6 (nucleotides 986-1043) contain the maximum heterogeneity which can provide the maximum discriminating power for the analysis of bacterial groups (Chakravorty et al., 2007; Shah et al., 2011).

This marker gene metagenomics is a fast and innovative way of obtaining a community distribution profile from the PCR amplification and sequencing of the 16S rRNA gene that can subsequently be associated with environmental data (metadata) derived from the sampling site under investigation (Oulas et al., 2015). Basically, this metagenomics approach was taken through the isolation of DNA from environmental samples, amplification of the collective 16S rRNA genes with degenerate PCR primer sets, subcloning the PCR products, and classification of the taxa present according to a database of assigned 16S rRNA sequences (Mardis, 2008).

CHAPTER 3: METHODOLOGY

3.1 Detection and isolation of bacteria associated with symptomatic bacterial wilt-diseased banana plants

3.1.1 Sampling sites

Samplings were carried out at five banana orchards in Batu Pahat, Johor (1°55'6.02"N 103°10'47.859"E); Teluk Chengai, Kedah (6°5'46.921"N 100°20'3.36"E); Bukit Temiang, Perlis (6°31'24"N 100°14'17.999"E); Kuala Pilah, Negeri Sembilan (2°44'25.706"N 102°14'55.938"E); and also Klang, Selangor (3°2'41.701"N 101°26'44.023"E). Approvals for doing the sampling was given by the officer in-charge from Crop Protection and Plant Quarantine Unit, Department of Agriculture (DOA) in each state. Those selected banana orchards were privately owned by the smallholders living in that area. At each sampling location, two to three symptomatic banana plants were chosen and chopped down with permission and assistance from the DOA workers and owner of the orchards (Table 3.1).

Most of the banana plants in these orchards showed symptoms of yellowing and wilting of leaves, forming a skirt of dead leaves around the pseudostem. In matured plant, fruits appeared unaffected on the external but internally, flesh was discoloured reddish-brown and rotted. Bacterial streaming test was conducted on site, as a rapid diagnostic tool for quick confirmation of bacterial wilt symptom (Muthoni et al., 2012). A cut portion of the pseudostem was immersed in a beaker of water and presence of bacterial ooze was observed. Samples of stems, roots, fruits and/or bacterial oozes were collected from symptomatic part of the banana plants and soil samples were also collected from rhizosphere of the same area. Samples of tissues and soil were placed in separate sterile

bags and labelled accordingly while bacterial ooze exuded from cut stems were collected in a small container with sterile distilled water (dH_2O).

Table 3.1: Details of sampling and plant materials.

Sampling Month	Sampling Location	Soil Type	Banana Cultivar (cv.)	Plant Materials
December 2011	Batu Pahat, Johor	Disturbed land (urban soil)*	cv. Nipah	Pseudostem
				Fruit
				Soil rhizosphere
			cv. Lilin	Pseudostem
				Fruit
				Soil rhizosphere
March 2012	Teluk Chengai, Kedah	Disturbed land (urban soil)	cv. Mas	Pseudostem
				Root
				Soil rhizosphere
			cv. Nangka	Pseudostem
				Plant root
				Soil rhizosphere
Jun 2012	Bukit Temiang, Perlis	Rasau series**	cv. Berangan	Pseudostem
				Fruit
				Bacterial ooze
October 2012	Kuala Pilah, Negeri Sembilan	Disturbed land (urban soil)	cv. Rastali	Pseudostem
				Fruit
				Bacterial ooze
			cv. Tanduk	Pseudostem
				Fruit
				Root
			cv. Nangka	Pseudostem
				Root
				Soil rhizosphere
				Bacterial ooze
March 2013	Klang, Selangor	Disturbed land (urban soil)	cv. Berangan	Pseudostem
				Bacterial ooze
				Soil rhizosphere
			cv. Nipah	Pseudostem
				Bacterial ooze
				Fruit

* Disturbed land (urban soil): material that has been manipulated, disturbed or transported by man's activities in the urban environment (Craul, 1992).

**Rasau series: Well-drained type of soil and the parent material is alluvial deposits (Gasim et al., 2011).

3.1.2 Rapid detection of *Ralstonia* species complex from bacterial wilt-diseased banana plant

A rapid diagnostic for the detection of *R. solanacearum* was conducted with the ImmunoStrip for Rs (AgDia, Inc., Elkhart, IN) according to the manufacturer's instructions. Prior to the ImmunoStrip test, positive (*R. solanacearum*) and negative (*Enterobacter* sp.) controls were prepared by culturing the bacterial isolates in CPG broth and incubated for 24 h (150 rpm in a room temperature). On the next day, bacterial suspensions were spectrophotometrically adjusted to 0.1 $OD_{600\ nm}$ with CPG broth. Fifty microliters (µl) of bacterial suspensions were mixed with 250 µl of BEB1 extraction buffer. For each infected plant sample, 250 µl of bacterial ooze collected from the stem was mixed with 250 µl of BEB1 extraction buffer. The provided strip was then inserted into each extract following the usage instructions and the strip was remained in the sample extract for 30 min before result was recorded.

Stem, root and fruit samples were then cut into small pieces, snap-frozen in liquid nitrogen (N_2) and stored in -80°C. DNA was then extracted from 200 mg tissue samples of infected banana plant using the method previously described by Rahman et al. (2010). Prior to DNA extraction, tissue samples were taken out from -80°C freezer, quickly ground into powder form with sterile mortar and pestle in liquid N_2. One millilitre (ml) extraction buffer (Appendix A) was used per 50 mg powder form plant tissues. Samples were incubated for 1 h at 55°C, followed by centrifugation at room temperature for 5 min.

Supernatant was transferred into a fresh 2 ml centrifuge tube. RNase A was added at a final concentration of 200 µg/ml prior to 1 h incubation at 37°C. An equal volume of phenol:chloroform:isoamylalcohol [25:24:1] was added, vortexed and centrifuged (5,000 rpm) at 4°C for 5 min. The upper aqueous phase was transferred into a fresh 2 ml centrifuge tube and equal volume of chloroform:isoamylalcohol [24:1] was added and

mixed followed by centrifugation (5,000 rpm) at 4°C for 5 min. Again, the upper aqueous phase was transferred into a fresh 2 ml centrifuge tube. Equal volume of ice-cold isopropanol was added, mixed and incubated at -80°C for 1 h. The sample was thawed at 4°C (without agitation) prior to centrifugation (8,500 rpm) at 4°C for 20 min. The supernatant was discarded and the pellet was carefully washed twice with 1 ml of 70% (v/v) ethanol. Each washing step was followed by centrifugation (5,000 rpm) at room temperature for 5 min. Finally, the pellet was air-dried and re-suspended in 30 µl of sterile distilled water. The DNA concentration and quality check was determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Polymerase Chain Reaction (PCR) with species-specific primers OLI-1/Y2 (5'-GGGGGTAGCTTGCTACCTGCC-3'/5'-CCCACTGCTGCCTCCCGTAGGAGT-3'), which target the specific 16S rRNA of *Ralstonia* species complex as described by Seal et al. (1993) was adopted. The PCR mixture contained 1X Buffer (Promega, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primers, 1U *Taq*Polymerase and approximately 50 ng of DNA template (Appendix B). The PCR conditions consisted of denaturation at 94°C (40 sec), annealing at 65°C (30 sec) and extension at 72°C (1 min) for 35 cycles. Amplicons were further purified and analysed by electrophoresis on l.5% (w/v) agarose gel and visualized under UV light (300 nm) after GelRed staining. Purified amplicons were submitted to a commercial company for sequencing and the results were analysed using the National Center for Biotechnology Information (NCBI) BLAST System (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.1.3 Procedure for bacterial isolation using cultural method

Tissue fragments (about 2 cm^2) were washed with 10% (v/v) sodium hypochlorite (NaClO) approximately for 2-3 min, then rinsed three times with sterile dH_2O. Tissues

were dried on filter paper. Later, surface-dried tissues were cut into smaller pieces. The tissue fragments were then macerated in 10 ml phosphate buffer solution (PBS) and left for 5-10 min. One ml of the bacterial suspension was serially diluted up to 10^{-4}. Twenty µl from dilution of 10^{-3} and 10^{-4} were cultured in duplicate on Tetrazolium-chloride (TZC) medium [dextrose 5 g/l, peptone 10 g/l, casamino acids 1 g/l, agar 18 g/l and 0.005% (w/v) of 2, 3, 5 triphenyl tetrazolium chloride] and semi-selective medium, South Africa (SMSA) [glycerol 5ml/l, peptone 10 g/l, casamino acids 1 g/l, agar 18 g/l, 0.005% (w/v) of TZC, 100 ppm Polymyxin B sulphate, 5 ppm crystal violet, 25 ppm bacitracin, 0.5 ppm penicillin and 5 ppm Chloramphenicol]. Furthermore, bacterial ooze were directly plated on TZC and SMSA media.

The collected soil samples were left to air-dry at room temperature for 2 days. About 10 g of sieved soil were added to an Erlenmeyer flask containing 100 ml of casamino peptone glucose (CPG) broth (modified from TZC medium by excluding triphenyl tetrazolium chloride) broth and 100 ml of SMSA enrichment broth. The samples were agitated overnight and serially diluted. 0.2 ml from dilutions (10^{-3} and 10^{-4}) were spread plated on SMSA medium and further purified on TZC medium. Well-isolated colonies showing the expected morphology (fluidal or mucoid, irregular shapes and surfaces, creamy-white in colour with pink- or red-pigmented centre) on TZC plates were selected and stored in 50% (v/v) glycerol stock, kept at -80°C for further analysis. All plates were incubated at 30 ± 2°C for 24 – 48 h.

Pure isolates were revived from stock culture by streaking onto non-selective Nutritive Agar (NA) and incubated overnight at 30 ± 2°C prior to biochemical tests such as Gram staining, 3% (w/v) potassium hydroxide (KOH) string test (confirmation test of the Gram-stain), catalase, oxidase, starch hydrolysis and arginine dihydrolase tests (Dhital et al., 2001). Isolates which have the characteristics of *R. solanacearum* (Gram negative,

catalase and oxidase positive, arginine dihydrolase negative, and inability to hydrolyse starch) were subjected to molecular identification technique.

3.1.4 Preparation of DNA template for Polymerase Chain Reaction

Genomic DNA was extracted from pure bacterial colonies using the NucleoSpin Tissue kit (MacHerey-Nagel, United States) according to manufacturer's protocols. Bacterial cells were harvested by centrifugation at 12, 000 rpm for 2 min and supernatant were discarded. Cells pellet was mixed thoroughly with 180 µl Buffer T1 and 25 µl Proteinase K. Bacterial samples were vortexed vigorously and incubated at 56°C until complete lysis (took around 2 – 3 h). Following incubation, samples were vortexed and 200 µl Buffer B3 was added to each sample. Samples were again vortexed vigorously and incubated at 70°C for 10 min. Samples were vortexed briefly and 210 µl absolute ethanol was added to each sample followed by vigorous vortexing. Each sample was then applied to the NucleoSpin Tissue column and centrifuged at 12, 000 rpm for 1 min. The washing step was done by adding 500 µl Buffer BW into the column and centrifuged again at 12, 000 rpm for 1 min. Following that, 600 µl Buffer B5 was added to the column and centrifuged at 12, 000 rpm for 1 min. To remove residual ethanol, the column was again centrifuged for 1 min and placed in a clean 1.5 ml micro centrifuge tube. Highly pure DNA was eluted by carefully pipetting 30 µl of Buffer BE into the centre of the column and incubated at room temperature for 2 min prior to centrifugation at 12, 000 rpm for 1 min.

3.1.5 Identification of bacterial isolates

Extracted DNA of putative bacterial colonies (Gram negative, catalase and oxidase positive, arginine dihydrolase negative, and inability to hydrolyse starch) were subjected

to PCR amplification using the species-specific, OLI-1/Y2 primers as previously described. Non-putative isolates were re-streaked onto CHROMagar Orientation^{TM} (CHROMagar, Paris, France) medium for further confirmation. This particular selective medium was used to help in differentiation of bacterial species based on different colony morphology due to its usefulness in isolating and identifying various bacterial species of *Enterobacteriaceae* family (Scarparo et al., 2002).

Subsequently, selected representative isolates were further tested with the universal 16SrRNA primers as described by Kajikazawa et al. (2007) with some modifications, 27f/1492r (5' AGAGTTTGATCCTGGCTCAG 3'/ GGTTACCTTGTTACGACTT 3'). 1µl of DNA template was added to 24µl of PCR mixture that consisted of 1X Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primers (27f/1492r), and 1U *Taq*Polymerase (Appendix B). PCR was performed with an initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s and a 72°C extension for 5 min. A *R. solanacearum* strain (GMI1000) received from University of Wisconsin-Madison, USA (isolated from BW-diseased tomato plant) was used as a positive control for PCR amplifications.

The 1500 base pair (bp) amplicons produced were subjected to gel electrophoresis on 1.5% (w/v) agarose gel at 100V for 30 min in Tris-borate buffer and visualized under UV light after GelRed staining. Sequencing the PCR products carried out validity test of the amplicons. The sequence data were analysed using the NCBI BLAST System.

3.2 Determination of biofilm-forming ability of selected bacteria isolated from symptomatic and non-symptomatic bacterial wilt-diseased banana plants

3.2.1 Sampling of symptomatic and non-symptomatic banana plants

In October 2013, the sixth sampling was carried out at the banana orchard in Kampung Sungai Burung, Tanjong Karang, Selangor (3°27'56.747"N 101°8'9.035"E). This subsequent sampling was conducted with a purpose of isolating bacterial communities using the non-selective medium from the symptomatic and non-symptomatic banana plants, which were selected from the same banana orchard.

A rapid test to detect *R. solanacearum* was conducted with ImmunoStrip for Rs (AgDia, Inc., Elkhart, IN) according to the manufacturer's instructions as previously described (sub-chapter 3.1.2). Samples of stems, roots and bacterial oozes from both symptomatic and non-symptomatic plants were processed as previously described. Diluted bacterial suspensions were spread-plated on CPG medium. Plates were incubated at 30 ± 2°C for 24 – 48 h. After two days, bacterial colonies harvested from the non-selective, CPG medium were re-streaked onto fresh CPG medium until purified colonies were obtained.

3.2.2 Identification of bacterial isolates

All purified isolates were subjected to 3% (w/v) potassium hydroxide (KOH) string test to distinguish between Gram-positive and Gram-negative bacteria. Gram-negative isolates were then streaked onto the CHROMagar Orientation™ (CHROMagar, Paris, France) medium. This medium was again used to differentiate bacterial isolates based on different colony morphology.

Following the previous step, the representative isolates from different colour morphotypes were tested with PCR targeting the V3-V5 region of the 16S rRNA gene as described by Muyzer et al. (1995) with some modifications. The PCR mixture contained 1X Buffer (Promega, USA), 1 mM $MgCl_2$, 0.1 mM dNTPs, 0.1 µM of the partial 16SrRNA primers, 357F/907R (CCTACGGGAGGCAGCAG/ CCGTCAATTCMTTTGAGTTT), 0.5 U *Taq*Polymerase and approximately 50 ng of DNA template (Appendix B). The PCR conditions consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C (1 min), annealing at 58°C (1 min 30 sec) and extension at 72°C (1 min) and finally further extension at 72°C for 5 min. PCR products were then analysed by electrophoresis on l.5% (w/v) agarose gel and visualized under UV light after GelRed staining. Amplicons were further purified, sequenced and the results were analysed using the NCBI BLAST System.

3.2.3 Biofilm-forming ability test for representative identified bacteria

The microtiter plate assay was used in this study to check for biofilm-forming ability based on Chelvam et al. (2014), with some modifications. Twelve selected bacterial strains were grown overnight in casamino peptone glucose (CPG) broth. Each bacterial suspension was spectrophotometrically standardised to 0.1 $OD_{600\ nm}$ for all experiments. These 12 bacterial suspensions were then inoculated into 96-well microtiter plate (eight wells for each strain) and incubated for 48 hrs. The blank control wells contained CPG broth only. After incubation, unbound cells and broth were carefully removed and adhered biofilms were heat fixed in an oven for 1 h at 60°C. Adhered biofilms were stained by addition of 200 µl of 0.1% (w/v) crystal violet for 5 min. The stain was removed by thorough washing with dH_2O. In order to quantify adhered cells, 200 µl of

95% (v/v) ethanol (decolouring solution) was added to each well. The absorption of the eluted stain was measured at $OD_{590\ nm}$.

Based on the optical density (O.D.) reading, strains were classified into the following categories: no biofilm formers, weak, moderate or strong biofilm formers, as previously described (Stepanovic et al., 2000). Briefly the cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. Strains were categorized into O.D. ≤ O.D.c = non-biofilm former, O.D.c < O.D. ≤ (2 × O.D.c) = weak biofilm former, (2 × O.D.c) < O.D. ≤ (4 × O.D.c) = moderate biofilm former and (4 × O.D.c) < O.D. = strong biofilm former. The blank control has been deducted from the bacterial strains O.D. readings. Experiments were performed in three independent times to ensure reproducibility.

3.3 Mono- and co-culture inoculations of biofilm-forming bacteria with blood disease pathogenic strain, *Ralstonia syzygii* subspecies *celebesensis* (BDB) into banana plants in glasshouse conditions

3.3.1 Bacterial culture and plant materials

Sixty-six 2-months old, tissue-cultured banana plants (*Musa acuminata* cv. Berangan) were acclimatized in a glasshouse, in polyethylene bags for 2 weeks. Positive control strain, *Ralstonia syzygii* subspecies *celebesensis* (Blood disease bacterium A2 HR-MARDI, Reference number: KF208537) (provided by MARDI Serdang, Selangor) inoculum was prepared by using fresh colonies of 48 h pre-prepared culture, into CPG broth, incubated at 150 rpm in a room temperature for 24 h. Four selected strong biofilm-formers were also cultured in CPG broth and incubated at 150 rpm in room temperature for 24 h. All bacterial suspensions were standardized to 1.0 x 10^7 cfu/ml (optical density

at 600 nm, 0.3) with CPG broth and was immediately used. Details of bacterial strains are shown in Table 3.2.

Table 3.2: Details of bacterial strains used in this study.

Bacterial strains	Bacterial Designation	Source	Biofilm-forming ability*
Enterobacter hormaechei	EH	Banana stem	strong
Kosakonia radicincitans	KR	Banana stem	strong
Enterobacter cloacae	EC	Banana stem	strong
Klebsiella pneumoniae	KP	Bacterial ooze	strong
Ralstonia syzygii subsp. *celebesensis*	BDB	from MARDI Serdang, Selangor	N/A**

*Biofilm-forming ability was previously tested with microtiter plate assay (Chelvam et al., 2014). Strong biofilm producers ($A_{590\ nm} > 1.3$).

**N/A – not available.

3.3.2 *In-vivo* assessment of mono- and co-culture inoculations of biofilm-forming bacteria with blood disease pathogen in banana plants

After 2 weeks of acclimatization, plants were inoculated with bacterial suspensions according to the BDB glasshouse inoculation technique developed by MARDI. Figure 3.1 showed the rows of acclimatized plants in the glasshouse.

Figure 3.1: Two months old banana plants as seven replicates for each treatment.

For each banana plant, 5 ml of inoculum were inoculated into the base of pseudostem (10 cm above soil level). All treatments including the controls were replicated seven times (n=7). The experiment was conducted in two sets for weekly observations and the disease severity index (DSI) experiments. Positive control plants were inoculated with 5 ml of *Ralstonia syzygii* subsp. *celebesensis* (BDB) suspension only while, the negative control plants were inoculated with 5 ml of CPG broth. Inoculated plants were maintained in polyethylene bags in Randomized Complete Block Design experiment (at 29°C ± 6°C in closed glasshouse, watered with tap water and allowed normal daylight). Details of treatments are shown in Table 3.3.

Table 3.3: Experimental design for the *in-vivo* assessment.

Treatment	**Treatment Designation**	**Bacterial suspension***
Treatment 1	T1	2.5 ml of KP + 2.5 ml of BDB
Treatment 2	T2	5 ml of KP
Treatment 3	T3	2.5 ml of EC + 2.5 ml of BDB
Treatment 4	T4	5 ml of EC
Treatment 5	T5	2.5 ml of KR + 2.5 ml of BDB
Treatment 6	T6	5 ml of KR
Treatment 7	T7	2.5 ml of EH + 2.5 ml of BDB
Treatment 8	T8	5 ml of EH
Positive control	T9	5 ml of BDB
Negative control	-VE	5 ml CPG broth

*KP = *Klebsiella pneumoniae*, EC = *Enterobacter cloacae*, KR = *Kosakonia radicincitans*, EH = *Enterobacter hormaechei*, BDB = *Ralstonia syzygii* subsp. *celebesensis*, CPG = Casamino peptone glucose. A standardized suspension of 1.0 x 10^7 cfu/ml (optical density at 600 nm, 0.3) was prepared for all bacterial cultures.

3.3.3 Observations of the external and internal symptoms

After inoculation, plants were monitored every week for the occurrence of any physical symptoms such as, wilting/epinasty, chlorosis and/or stunting. When some of the inoculated plants started to produce physical symptoms, a plant from each treatment, with

and without symptoms were dissected vertically to observe any appearance of dark brown stripes in the vascular bundles of the banana stems, rhizomes and roots.

Scales for external symptoms index (ESI) and internal symptoms index (ISI) were also given weekly based on the severity level of each of the seven replicated plant according to external and internal observations, respectively (Figure 3.2 and 3.3).

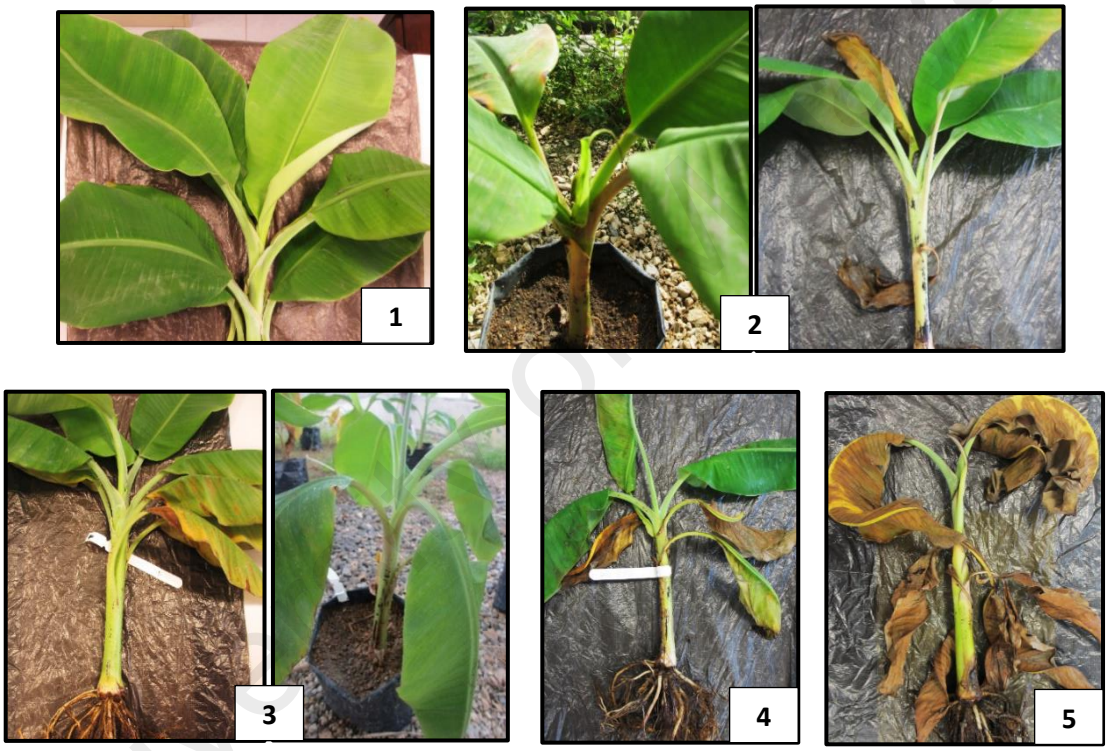

Figure 3.2: External Symptoms Indices (ESI)

Scales	Indications
1	No symptoms/ Plants appear healthy
2	Stunted leaves/ Weird leaves arrangement
3	Light wilt/ Epinasty
4	Wilting (Partly wilt)
5	Total wilt/ Dying

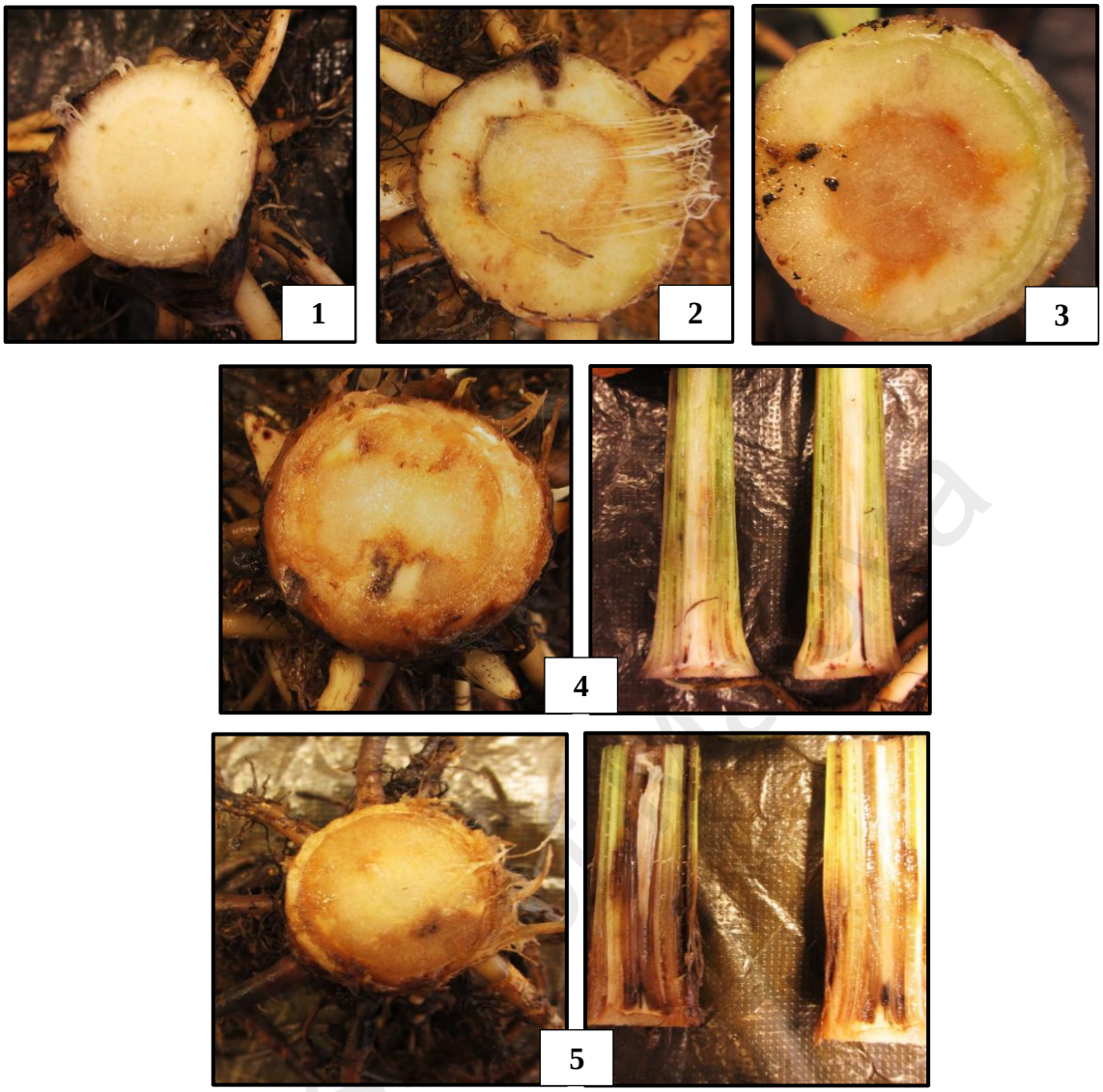

Figure 3.3: Internal Symptoms Indices (ISI)

Scales	Indications
1	No discoloration of tissues of rhizome and pseudostem
2	Less than 50% of rhizome discoloured
3	More than 50% of rhizome discoloured
4	Discoloration of entire rhizome and brown stripes in pseudostem
5	Discoloration of entire rhizome and pseudostem

For each given ESI and ISI scales, disease severity index (DSI) was calculated and interpreted. The overall DSI score was calculated and interpreted based on the modified formula of Fusarium wilt study by Mak et al. (2001):

$$\text{DSI} = \frac{\sum (\text{Number on scale} \times \text{Number of plants in that scale})}{\sum (\text{Number of inoculated plants})}$$

Interpretation for DSI scores for each treatment are listed in Table 3.4. Four designation for DSI scores, namely resistant, tolerant, susceptible, and highly susceptible. The final status of disease expression for plants under each treatment follows the more severe DSI scores results. For instance, if the plant is tolerant in ESI and resistant in ISI, then the plant is considered as tolerant. If any one of the ESI or ISI result is highly susceptible, then the final status for the treatment is highly susceptible. If both ESI and ISI are tolerant, the plant is indicated as tolerant.

Table 3.4: Interpretation of Disease Severity Index (DSI) scores.

DSI scores for ESI	**DSI scores for ISI**	**Interpretation**
1	1	Resistant
1.1 to 2	1.1 to 2	Tolerant
2.1 to 3	2.1 to 3	Susceptible
3.1 to 4	3.1 to 4	Highly susceptible

3.3.4 Re-isolation and identification of bacterial species from the inoculated banana plants

Fortnightly, re-isolation of bacteria from the cut plants from each treatment was conducted to detect the presence of the inoculated bacteria in the treated banana plants. Prior to the isolation technique, pieces of stem, rhizome and root tissues were randomly cut into small pieces.

Plant tissues were surface-sterilized as previously described (sub-chapter 3.1.3) (Figure 3.4). Ten ml of sterile dH_2O were added and tissues were ground with mortar and pestle. One ml of the filtrate were serially diluted up to 10^{-4}. Twenty µl from dilution of 10^{-3} and 10^{-4} were plated on TZC and semi-selective, SMSA media. Bacterial colonies that appeared on plates were further sub-cultured onto CPG medium. At the final observation week (8^{th} WPI), the re-isolated bacterial colonies from a plant in each treatment were subjected to PCR amplification technique, in order to detect and confirm the presence of the inoculated bacteria in those treated plants.

Figure 3.4: Tissue samples preparation prior to bacterial isolation.

3.3.4.1 Preparation of DNA template for PCR technique

Pure isolated bacterial colonies were grown overnight in CPG broth on a rotary shaker at 32°C. DNA extraction was carried out by using the DNeasy Blood and Tissue kit (Qiagen, Germany) according to the manufacturer's protocols (Appendix C). The extracted DNA was subjected to PCR amplification.

3.3.4.2 *R. solanacearum* species complex primers

Identification of BDB was done by using *R. solanacearum* species complex primers, 759/760 (detect *R. solanacearum* and *R. syzygii* complex strains). These primers were used to amplify a ~282-bp fragment (Villa et al., 2003).

The extracted DNA was amplified in 50 µl reaction volumes containing 25 µl PCR Master Mix, Thermo Scientific DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, USA), 1 µM of each primer, 759/760 (5'-GTCGCCGTCAACTCACTTTCC-3'/5'-GTCGCCGTCAGCAATGCGGAATCG-3') and 1 µg of DNA template. The thermal cycler consisted of initial denaturation at 94°C for 30 sec; 40 cycles at 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min; followed by a 72°C extension for 10 min in MyCycler™ Thermal Cycler (BioRad Laboratories Inc., USA). All PCR products were analysed on 1% (w/v) agarose gel at 80V for 90 min in Tris-borate buffer. Gel stained with FloroSafe DNA Stain (1st Base, Singapore) and visualized by Compact Digimage System UVDI (Major Science, USA).

3.3.4.3 16S rRNA primers

Identification of other bacterial isolates was done by using the 16S rRNA sequencing with 357F/907R primers (amplified the V3-V5 region of the 16S rRNA gene) as previously described (sub-chapter 3.2.2). Amplicons were analysed on 1% (w/v) agarose gel at 80V for 90 min in Tris-borate buffer and visualized by Compact Digimage System UVDI (Major Science, USA). The amplicons were further purified with QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instruction (Appendix C). Purified amplicons were sent for sequencing and analysed using NCBI BLAST System.

3.3.5 Preparation of pseudostems' tissues for the scanning electron microscopic analysis

To examine the structural changes of plant tissues in the stems of inoculated banana plants, a plant from each treatment were removed from the polyethylene bags after two months inoculation (8^{th} WPI). The roots and top parts of the plants were cut off and the remaining stems were washed to remove dirt. The remaining parts were cross sectioned about 1 – 2 cm length prior to preparation for Scanning Electron Microscopic (SEM) analysis. Few small portions of the rhizome tissues were fixed with 8% (v/v) Glutaraldehyde in 0.2 M Phosphate Buffer (Sorensen's), mix at a ratio of 1:1 at room temperature for 1 h. After fixation, the tissue was washed with 0.1 M Phosphate Buffer (Sorensen's) and then covered with 4% (v/v) Osmium tetra-oxide in dH_2O, incubated at room temperature for 14 h.

On the next day, fixed stem tissues were washed with dH_2O. Subsequently, the tissues were dehydrated in ascending series of different percentage of ethanol, [10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 100% (v/v)] for 15 min each, followed by ethanol-acetone mixture, [in a ratio of ethanol to acetone (3:1, 1:1 and 1:3)] for 20 min each and finally four times in pure acetone for 20 min each. These dehydration steps were followed by critical point-drying (CPD) of the tissue samples, then, mounted on scanning electron micrograph stubs, sputter-coated with gold using a table-top sputter coater (Model: Leica EM SCD005) and finally observed under the Scanning Electron Microscope (Model: JEOL JSM-7001F).

3.3.6 Statistical analysis

The ESI and ISI data were finally subjected to statistical analysis using the IBM SPSS Statistics software. Nonparametric tests for two or more independent samples was performed and Kruskal-Wallis 1-way ANOVA was chosen to compare distributions across the treatments. The significance level was set at 0.05.

3.4 Whole genome sequence of *Kosakonia radicincitans*, a biofilm-forming bacterial strain associated with bacterial wilt-diseased *Musa* spp. plant

3.4.1 Whole genome sequence

The bacterial strain was cultured overnight in a 15 ml tube containing Luria Bertani broth at 32°C. Genomic DNA was extracted from *K. radicincitans* UMEnt01/12 by using the DNeasy Blood and Tissue kit according to the manufacturer's instructions (Qiagen, Germany) as described in Appendix C. Genomic DNA was subjected to quality ($A_{260/280}$ and $A_{260/230}$) and quantity check with Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The high-quantity pure genomic DNA was further analysed with gel electrophoresis on 1% (w/v) agarose gel at 100V for 30 min in Tris-borate buffer and visualized under UV light (300nm) after GelRed staining.

The whole genome sequencing was performed at a commercial facility (ScienceVision Sdn. Bhd., Malaysia) using the Illumina Hiseq2000. The 150 bp paired-end sequencing was done with an insert size of 300 bp. About 1.6 million reads and 580 Mb of data were obtained with 37x genome average coverage was achieved.

3.4.2 Genome annotation and analysis

The resulting sequence data were quality assessed, trimmed and assembled with Velvet using *de novo* approach in the same way as described by Yap et al. (2012), into 71 contigs by using CLCBio Genomic Workbench 5.1 (CLCBio, Denmark). Scaffolding of the pre-assembled contigs using paired-read data was performed by SSPACE (SSAKE-based Scaffolding of Pre-Assembled Contigs after Extension) version 20 scaffolder. Gapfiller version 1.10 was used to fill up the gaps within these scaffolds by aligning the reads against already generated Scaffolds by SSPACE. After manual curation of the final draft of nucleotide sequences, annotation was carried out using the SEED Viewer (http://pubseed.theseed.org/) and the Rapid Annotation using Subsystem Technology (RAST) version 2.0 (http://rast.nmpdr.org/) (Overbeek et al., 2014), also the Blast2GO (https://www.blast2go.com/) annotation server (Conesa et al., 2005). The genome statistics were gleaned using Artemis (http://www.sanger.ac.uk/Software/Artemis) (Rutherford et al., 2000). The tRNA and rRNA genes were identified with RNAmmer 1.2 (Lagesen et al., 2007) and tRNAscan-SE 1.21 (Lowe & Eddy, 1997) server, respectively. Genome sequence of *K. radicincitans* UMEnt01/12 was compared to the genome of *K. radicincitans* DSM16656^{T} by using pan-genome analysis pipeline (PGAP). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequence was identified using CRISPR Finder (http://crispr.u-psud.fr/Server/CRISPRfinder.php) (Grissa et al., 2007).

Phylogenetic analyses were performed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.2 (http://www.megasoftware.net/) (Tamura et al., 2011) after multiple alignment of data by ClustalW. Distances (distance options according to the Kimura-2 model) and clustering with the Neighbor-Joining method and maximum parsimony was performed by using Bootstrap values based on 1000 replications.

GeneBank Accession Number: This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number JDYJ00000000. The BioProject designation for this project is PRJNA236629.

3.5 16S rRNA metagenomics sequencing and analysis of symptomatic and non-symptomatic bacterial wilt-diseased banana plants

3.5.1 Plant materials

Banana orchards of the smallholder cropping system infected with BWD were identified in two locations in Negeri Sembilan and Selangor, Malaysia. A non-symptomatic (NS) and a symptomatic (SA) banana plants (*Musa paradisiaca* cv. Nipah) were selected from an orchard in Kuala Pilah, Negeri Sembilan (2°44'25.706"N 102°14'55.938"E), while another symptomatic (SB) banana plant also from cv. Nipah was selected from an orchard in Kuala Selangor, Selangor (3°20'24.662"N 101°14'59.143"E). Samples of pseudostem parts were collected from each banana plants, placed in sterile plastic bags and brought to the laboratory. Pseudostem tissues were labelled accordingly (Table 3.5).

Table 3.5: Source and types of plant samples.

Sampling location	Soil type	Type of plant	Sample material	Sample designation
Kuala Pilah, Negeri Sembilan	Disturbed land*	Non-symptomatic	Pseudostem	NS
		Symptomatic A		SA
Kuala Selangor, Selangor		Symptomatic B		SB

*Disturbed land (urban soil): material that has been manipulated, disturbed or transported by man's activities in the urban environment (Craul, 1992).

In the laboratory, pseudostem tissues from both banana plants were crushed and subjected to ImmunoStrip for Rs (AgDia, Inc., Elkhart, IN) test according to the manufacturer's instructions. Approximately 0.15 g of tissues from both banana plant samples were cut into small pieces. Each tissue sample was placed between the mesh linings of the provided extract bag containing BEB1 extraction buffer which was labelled accordingly. The surface of the bag was rubbed with blunt side of the knife to grind and mix the sample. The strips were then inserted into each extract bag following the usage instructions and the strips were remained in the sample extracts for 30 min before results were recorded.

3.5.2 Preparation of total genomic DNA

Pseudostem tissues were also cut into small pieces (about 2 cm^2) and surface-sterilized with 10% (v/v) sodium hypochlorite (NaClO) approximately for 2 – 3 min. Tissue fragments were then rinsed three times with sterile dH_2O, dried on filter paper and finally wrapped with aluminium foil. Wrapped-tissue fragments were snap-frozen in liquid nitrogen and stored in -80°C. Prior to DNA extraction, the frozen tissue samples were quickly ground into powder form by using sterile mortar and pestle in liquid N_2.

Initially, DNA isolation was carried out according to protocol described by Rahman et al. (2010). However, for high quality and quantity of total genomic DNA, the extraction was carried out according to modified methods from Fan and Gulley (2001) and Souza et al. (2012). Note that 50 mg (fine powder) plant tissue sample was used for each DNA extraction tube.

3.5.2.1 CTAB DNA extraction protocol

DNA extraction was performed according to methods by Rahman et al. (2010). This protocol was described as an efficient method for removing PCR inhibitors such as plant and soil inhibitory components. The method utilizes cetyl trimethyl ammonium bromide (CTAB) as a detergent and phenol, which is an organic solvent for lipids and protein removal. Chloroform is then used to facilitate the removal of phenol. DNA is subsequently concentrated and further purified by precipitation in a cold isopropanol and ethanol. Finally, DNA is re-solubilized in sterile dH_2O.

3.5.2.2 Modified SDS-based DNA extraction protocol

DNA extraction was also carried out using methods modified from Fan and Gulley (2001) and Souza et al. (2012). One ml of sorbitol buffer (100 mM Tris-HCl, pH 8.0, 0.35 M sorbitol, 5 mM EDTA, pH 8.0, stored at 4°C, 1% PVP-40 with 1% 2-mercaptoethanol) was added to each plant tissues sample. Tissue sample was centrifuged at 6, 000 rpm for 10 min. Supernatant was discarded and cleaned up step with sorbitol buffer was repeated twice to three times. After centrifugation and supernatant removal, pellet was dissolved in 1.5 ml extraction buffer (10% sodium dodecyl sulfate (SDS), 10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA) followed by addition of 30 µl Proteinase K solution (10 mg/ml of proteinase K in 50 mM Tris-HCl, pH 7.5) and four microliter RNase A (20 mg/ml). The mixture was vortexed vigorously for few seconds prior to incubation at 65°C for 30 min (three times vortexing). After incubation, 130 µl sodium acetate (3M, pH 5.2) was added and incubated at 4°C for 10 min, followed by centrifugation at 13,000 rpm for 15 min. The supernatant was transferred to a new tube, 650 µl cold isopropanol was added. After gentle mixing, the sample was incubated at -20°C for 1 h. Following that, the sample was subjected to centrifugation (8,500 rpm, 4°C) for 30 min. The supernatant

was discarded while the pellet was carefully washed twice with 650 µl ice cold ethanol. After each washing step, the sample was centrifuged (13,000 rpm, 4°C) for 5 min. Finally, the DNA pellet was air-dried and resuspended in 30 µl sterile dH_2O. DNA was quantified by using Qubit™ 2.0 Fluorometer (Invitrogen, USA) and qualified ($A_{260/280}$ and $A_{260/230}$) with Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) prior to amplification technique.

3.5.3 Amplification of bacterial 16S rRNA and Illumina MiSeq sequencing

The DNA concentration was standardized to 10 ng/µl and 1 µl was used for each reaction. 16S rRNA v3 region (Figure 3.5) was amplified by using PCR technique with KAPA HiFi HotStart ReadyMix PCR Kit (KAPA BioSystems, Boston, MA, USA) and each sample was triplicate to obtain sufficient yield of DNA products. PCR master mix containing the appropriate volume of all reaction components was summarized in Appendix B. The PCR master mix, primers (Appendix D) and DNA template were transferred to individual PCR tubes, briefly mixed and centrifuged. The PCR amplification was performed according to the standard protocol which consist of initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 98°C (40 sec), annealing at 65°C (15 sec) and extension at 72°C (15 sec) and finally further extension at 72°C for 1 min. Then, the PCR amplified products were electrophoresed in 1.5% (w/v) agarose gel and visualized using GelStar™ nucleic acid gel staining (Lonza, USA). Gel extraction and purification were done with QIAquick PCR Purification Kit (Qiagen, Germany) according to manufacturer's instruction. The purified 16S rRNA amplicons were processed for sequencing-library preparation with the normalize and pool libraries step in TruSeq DNA sample preparation kit (Illumina, California, USA), following the

sample preparation guide prior to next generation sequencing (NGS) (2 × 151 bp paired end run) with MiSeq platform (Illumina, California, USA).

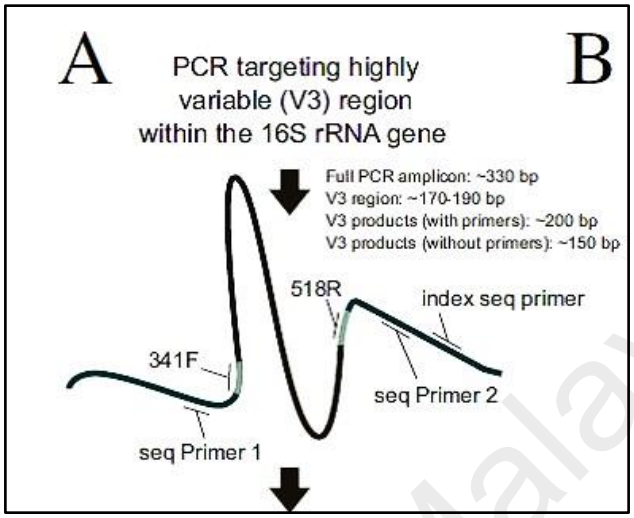

Figure 3.5: The schema indicating a PCR (20 cycles) of ~330-base amplicons, including the conserved 16S rRNA gene primer-binding region. Source of photo: Bartram et al. (2011)

3.5.4 Sequence analysis and taxonomic classifications

Millions of raw reads generated from Miseq with optimum cluster density were channelled to the CLC Genomic Workbench 7.0.4 (Qiagen, Germany) for ambiguous nucleotide trimming and bad reads filtered at the limit of Q20. Prior to these steps, quality control checked on raw sequences dataset of each sample was performed using FastQC (Andrews, 2012). In downstream analyses, the processed sequences were searched against publicly NCBI 16S Microbial database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLASTN to identify the microbial diversity. The NCBI is a larger database compared to other common databases (i.e., RDP and SILVA) (Chan et al., 2015). The outputs were then imported into Metagenome Analyzer (MEGAN4) version 4.64.2 (Huson et al., 2011) for taxonomical classifications. This software calculated the

taxonomic classification by mapping reads onto different taxa with the reference from NCBI taxonomy. Microbial abundance was assessed by counting the number of reads. Diversity indices (Simpson's and Shannon-Wiener) and species richness were calculated using MEGAN4 software. Rarefaction curves were also created using the same software.

Nucleotide Sequence Accession Number: The sequencing output files from this metagenomics project has been deposited at DDBJ/EMBL/GenBank under the accession number SRP056352. The BioProject designation for this project is PRJNA277904.

CHAPTER 4: RESULTS

4.1 Isolated bacteria associated with symptomatic bacterial wilt-diseased banana plants

Sampling was first conducted by surveying for banana orchards in five different states (Johor, Kedah, Perlis, Negeri Sembilan and Selangor) in Peninsular Malaysia that were reported to have cases of bacterial wilt disease of banana (Figure 4.1). The five banana orchards selected for sampling purposes were determined by the officer in-charge from Department of Agriculture (DOA) based on their surveys done on banana wilt-infected area. Details for the sampling locations and plant materials collected are described in Table 3.1.

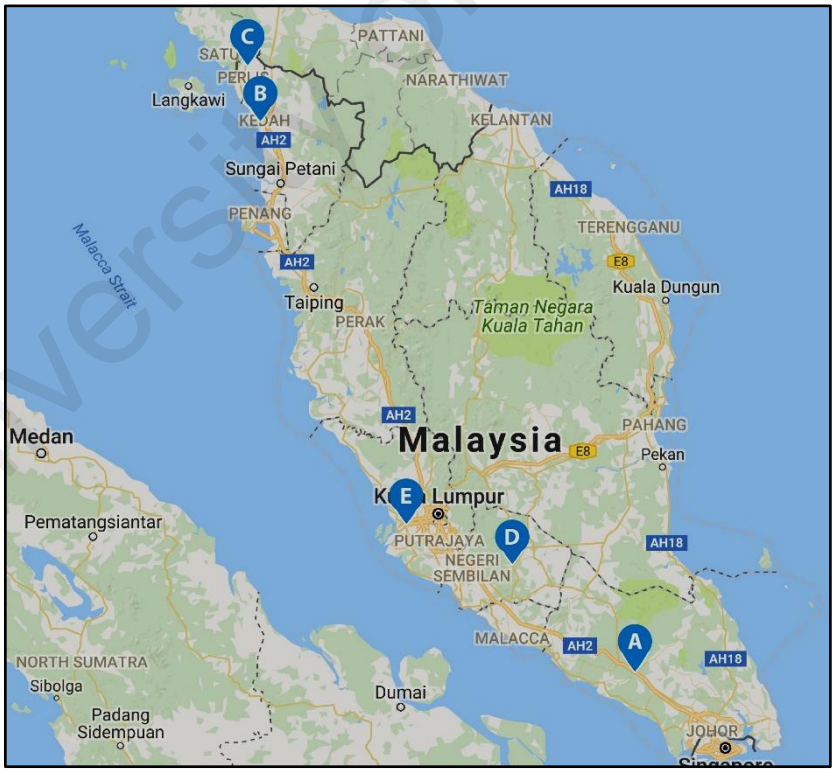

Figure 4.1: Plant sampling points in Peninsular Malaysia, A) Batu Pahat, Johor, B) Teluk Chengai, Kedah, C) Bukit Temiang, Perlis, D) Kuala Pilah, Negeri Sembilan, and E) Klang, Selangor.

The ten banana plants from different cultivars with typical disease symptoms were selected from these five orchards and chopped off to collect sample materials. Bacterial streaming test conducted showed that in 5 to 10 min, milky strands of bacterial ooze was observed flowing from the cut surface into the water (Figure 4.2).

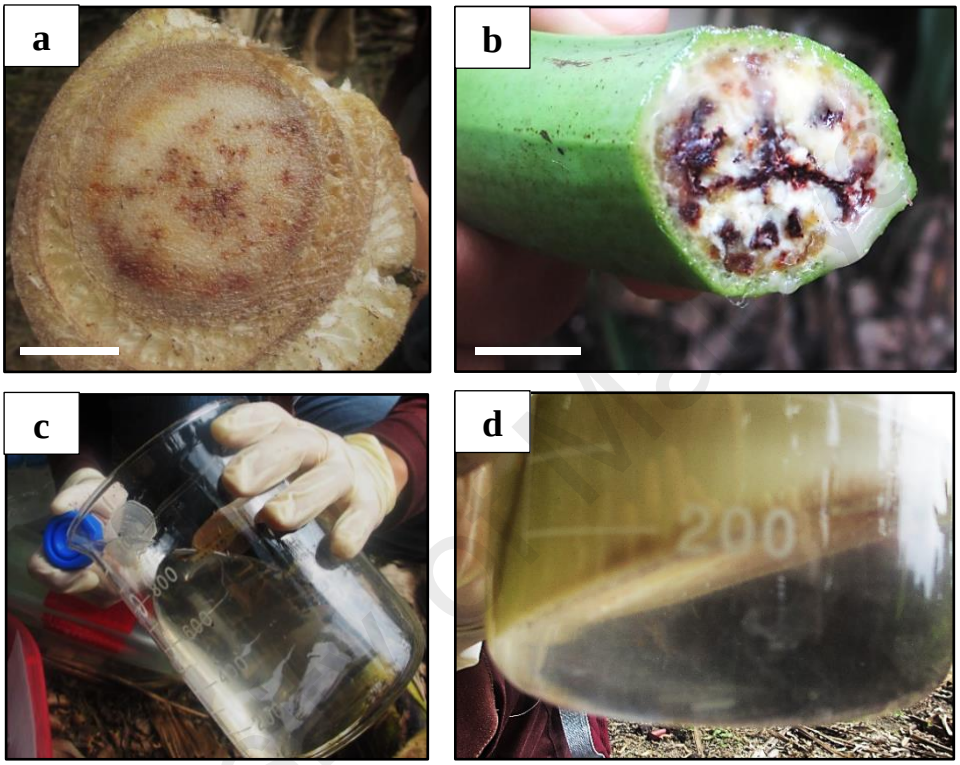

Figure 4.2: Samples of plant materials, a) pseudostem b) fruit and c) bacterial ooze collected from the infected banana plant. d) Milky strands of bacterial ooze can be observed exuded from the cut surface of banana pseudostem (scale bar, 5 cm and 1 cm)

4.1.1 Presence of *Ralstonia solanacearum* species-complex in bacterial wilt-diseased banana plant based on serological and molecular approaches

The ooze samples collected from pseudostems of ten BW-diseased banana plants (from five banana orchards) were tested directly with the ImmunoStrip which is specific for *R. solanacearum*. Nine out of ten (only the ooze samples from banana cv. Mas from Teluk Chengai, Kedah showed negative result) of the tested ooze samples gave positive results

for the presence of *R. solanacearum* as indicated by the appearance of control line and test line on the strips. Figure 4.3 shows example of the results for the ImmunoStrip test of ooze sample from an infected plant which was compared with negative control (*Enterobacter* sp. culture) and positive control (*R. solanacearum* culture). The ImmunoStrip test is the most rapid and least cumbersome means of screening plants with *R. solanacearum* infections (Paret et al., 2010).

Total genomic DNA directly extracted from tissue samples of infected banana plants were also analysed for the presence of *R. solanacearum* with *Ralstonia* sp. complex-specific primers, OLI-1/Y2. After the DNA amplification and gel electrophoresis, all of the extracted DNA from plant tissue (pseudostem, leaves and fruit) samples produced the desired bands of approximately 288 bp upon visualization under the UV light (Figure 4.4).

The amplicons were validated by DNA sequencing and the resulted sequence showed 99% sequence similarities with *Ralstonia syzygii*, 16S ribosomal RNA, partial sequence (Accession no. NR_134149.1) (members of *R. solanacearum* species-complex). Results for rapid detection were summarized in Table 4.1. Based on these two rapid detection methods, *Ralstonia solanacearum* was presumably present in those symptomatic banana plants samples. Hence, isolation technique was carried out using the plant tissues (pseudostem, leaves, root and/or fruit), bacterial oozes and soil rhizosphere samples collected from the five banana plantations infected with BW disease.

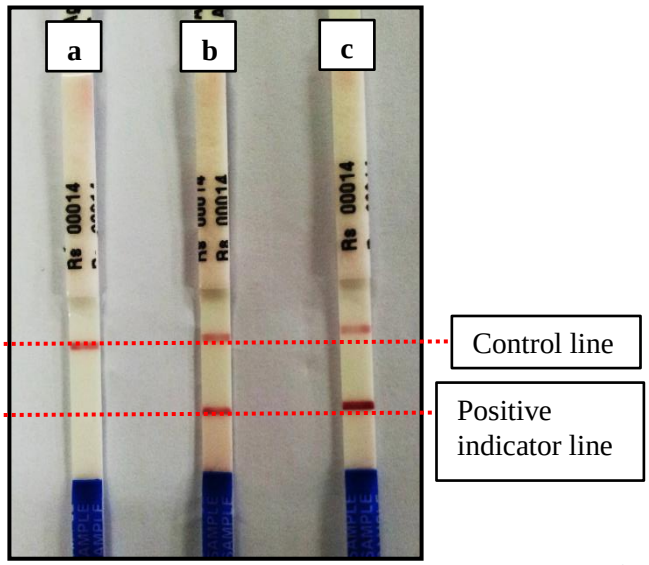

Figure 4.3: The infected plants screened for *R. solanacearum* using ImmunoStrips. a) Negative control, b) Positive control, c) Ooze from an infected plant sample.

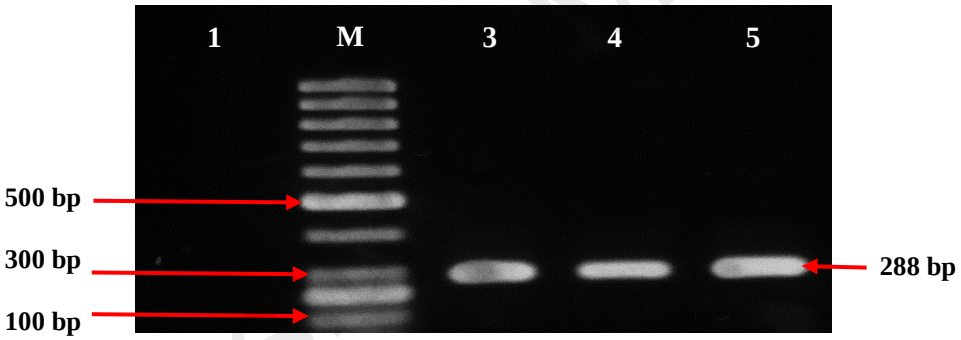

Figure 4.4: The representative gel electrophoresis of PCR amplification with OLI-1/Y2 primers.

Approximately 288 bp bands indicate the presence of DNA belonging to the *R. solanacearum* species-complex in the samples. 1, -ve control; M, 100 bp molecular size markers; 3, pseudostem tissue; 4, leaves tissue; 5, fruit tissue.

Table 4.1: Summarized results for rapid detection method.

Sampling Location	Banana Cultivar (cv.)	ImmunoStrip test*	Amplification of *Ralstonia* sp.-specific gene**
Batu Pahat, Johor	cv. Nipah	+	+
	cv. Lilin	+	+
Teluk Chengai, Kedah	cv. Mas	-	+
	cv. Nangka	+	+
Bukit Temiang, Perlis	cv. Berangan	+	+
Kuala Pilah, Negeri Sembilan	cv. Rastali	+	+
	cv. Tanduk	+	+
	cv. Nangka	+	+
Klang, Selangor	cv. Berangan	+	+
	cv. Nipah	+	+

* Positive (+) results for the presence of *R. solanacearum* as indicated by the appearance of control line and test line on the strips. Negative (-) result for the presence of *R. solanacearum* due to the absence of test line on the strips.

** Positive (+) results for the presence of *Ralstonia* sp. due detection of desired bands of approximately 288 bp upon visualization under the UV light and confirmation by sequencing (Seal et al., 1993).

4.1.2 Bacterial colonies obtained from semi-selective media

Bacterial colonies were obtained from the Tetrazolium-chloride (TZC) and semi-selective medium, South Africa (SMSA) agar after 24 – 48 h incubation at 28 ± 2°C (Figure 4.5). After further purification on TZC medium, 76 bacterial colonies were successfully isolated from a total of 32 samples of plant tissues, bacterial ooze and soil rhizosphere. These isolated bacterial colonies were fluidal and mucoid with typical irregularity of their surfaces, creamy-white in colour with pink- or red-pigmented centre (Figure 4.6). Biochemical test results for all the 76 bacterial isolates are recorded in Table 4.2.

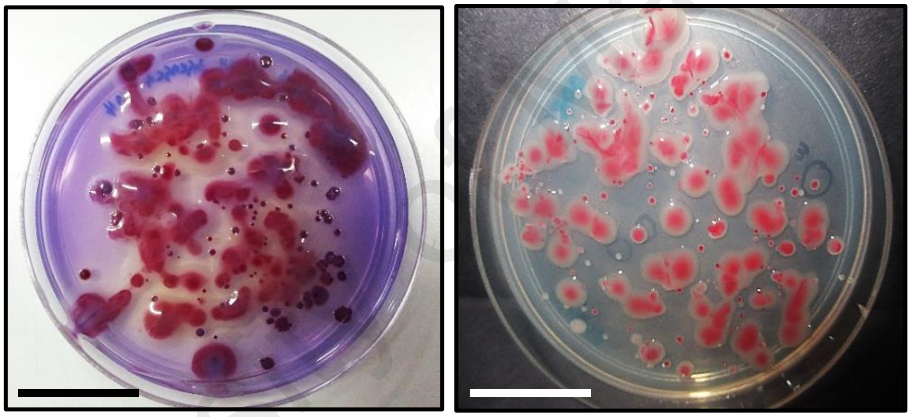

Figure 4.5: Isolated bacterial colonies on a) SMSA and b) TZC agar (scale bar, 3 cm)

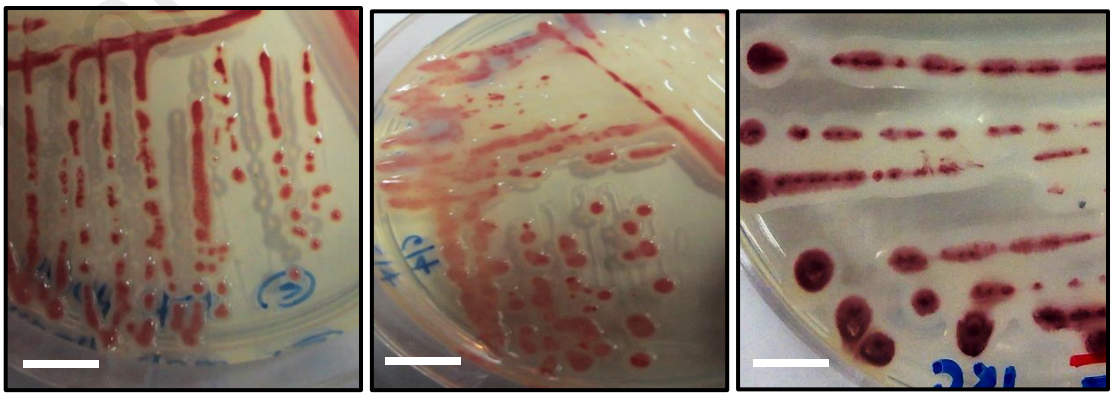

Figure 4.6: Colony phenotypes of three purified isolates on TZC agar (scale bar, 1 cm)

Table 4.2: Summary of biochemical reactions of 76 bacterial isolates from five sampling locations

Isolates Designation	Gram-Staining	Catalase	Oxidase	Starch Hydrolysis	Arginine Dihydrolase	*Expected ID
BP-LN-ST-1	positive	positive	negative	negative	negative	negative
BP-LN-ST-2	negative	positive	negative	negative	negative	negative
BP-LN-F1	negative	positive	negative	negative	negative	negative
BP-LN-F2	negative	positive	negative	negative	negative	negative
BP-LN-SL1	negative	positive	negative	negative	negative	negative
BP-LN-SL2	negative	positive	negative	negative	negative	negative
BP-LN-SL3	negative	positive	negative	negative	negative	negative
BP-NI-ST1	negative	positive	positive	negative	negative	positive
BP-NI-ST2	negative	positive	negative	negative	positive	negative
BP-NI-F1	negative	positive	negative	negative	negative	negative
BP-NI-F2	negative	positive	negative	negative	negative	negative
BP-NI-SL1	negative	positive	negative	negative	negative	negative
BP-NI-SL2	negative	positive	positive	negative	negative	positive
BP-NI-SL3	negative	positive	negative	negative	positive	negative
TC-NA-ST1	negative	positive	negative	negative	negative	negative
TC-NA-ST2	positive	positive	negative	negative	negative	negative
TC-NA-RT1	negative	positive	negative	negative	negative	negative
TC-NA-RT2	positive	positive	negative	negative	negative	negative
TC-NA-SL1	positive	positive	negative	negative	positive	negative
TC-NA-SL2	positive	positive	negative	negative	positive	negative
TC-MS-ST1	negative	positive	negative	negative	negative	negative
TC-MS-ST2	negative	positive	positive	negative	negative	positive
TC-MS-RT	negative	positive	positive	negative	negative	positive
TC-MS-SL1	positive	positive	negative	negative	positive	negative
TC-MS-SL2	negative	positive	negative	negative	positive	negative
TC-MS-SL3	negative	positive	negative	negative	negative	negative
BT-BR-ST1	positive	positive	negative	negative	negative	negative
BT-BR-ST2	negative	positive	positive	negative	negative	positive
BT-BR-ST3	negative	positive	positive	negative	negative	positive
BT-BR-ST4	positive	positive	positive	negative	negative	negative
BT-BR-ST5	negative	positive	negative	negative	negative	negative
BT-BR-F1	negative	positive	negative	negative	negative	negative
BT-BR-F2	negative	positive	negative	negative	negative	negative
BT-BR-F3	negative	positive	negative	negative	negative	negative
BT-BR-F4	negative	positive	negative	negative	negative	negative
BT-BR-O1	negative	positive	negative	negative	negative	negative
BT-BR-O2	negative	positive	negative	negative	negative	negative
BT-BR-O3	negative	positive	negative	negative	negative	negative
BT-BR-O4	negative	positive	negative	negative	negative	Negative
BT-BR-O5	negative	positive	negative	negative	negative	negative

Table 4.2, continued:

Isolate Designation	Gram-Staining	Catalase	Oxidase	Starch Hydrolysis	Arginine Dihydrolase	*Expected ID
BT-BR-O6	negative	positive	negative	negative	negative	negative
KP-NA-ST	negative	positive	negative	negative	negative	negative
KP-NA-RT1	negative	positive	positive	negative	negative	positive
KP-NA-RT2	negative	positive	positive	negative	negative	positive
KP-NA-SL1	negative	positive	negative	negative	negative	negative
KP-NA-SL2	negative	positive	positive	negative	negative	positive
KP-NA-O1	negative	positive	positive	negative	negative	positive
KP-NA-O2	negative	positive	negative	negative	negative	negative
KP-NA-O3	positive	positive	negative	negative	negative	negative
KP-RL-F1	negative	positive	negative	negative	negative	negative
KP-RL-F2	negative	positive	negative	negative	negative	negative
KP-RL-ST1	positive	positive	negative	negative	negative	negative
KP-RL-ST2	positive	positive	negative	negative	negative	negative
KP-RL-ST3	negative	positive	negative	negative	negative	negative
KP-RL-O1	negative	positive	positive	negative	negative	positive
KP-RL-O2	negative	positive	negative	negative	negative	negative
KP-RL-O3	positive	positive	negative	negative	negative	negative
KP-TD-ST1	negative	positive	positive	negative	negative	positive
KP-TD-ST2	negative	positive	positive	negative	positive	negative
KP-TD-F	negative	positive	negative	negative	positive	negative
KP-TD-RT1	positive	positive	negative	negative	negative	Negative
KP-TD-RT2	negative	positive	negative	negative	negative	negative
KG-BR-ST1	positive	positive	positive	negative	negative	negative
KG-BR-ST2	negative	positive	positive	negative	positive	negative
KG-BR-O1	positive	positive	positive	negative	negative	negative
KG-BR-O2	positive	positive	positive	negative	negative	negative
KG-BR-O3	negative	positive	positive	negative	negative	positive
KG-BR-SL1	positive	positive	negative	negative	negative	negative
KG-BR-SL2	positive	negative	negative	negative	negative	negative
KG-BR-SL3	negative	positive	positive	negative	negative	negative
KG-NI-ST1	positive	positive	positive	negative	positive	negative
KG-NI-ST2	negative	positive	positive	negative	negative	positive
KG-NI-O1	negative	positive	positive	negative	negative	positive
KG-NI-O2	negative	positive	negative	negative	negative	negative
KG-NI-O3	negative	positive	negative	negative	negative	negative
KG-NI-F1	negative	positive	negative	negative	negative	negative

*Expected ID is positive only if the isolate was Gram-negative, catalase positive, oxidase positive, arginine dihydrolase negative and was unable to hydrolyse starch.

Among the 76 isolates tested, only 15 fulfilled the characteristics of putative *R. solanacearum* which were Gram-negative, catalase and oxidase positive, arginine dihydrolase negative, and also was unable to hydrolyse starch. Meanwhile, another 45 of the bacterial isolates were also Gram-negative, catalase positive, arginine dihydrolase negative, and were unable to hydrolyse starch but oxidase negative. These indicated few biochemical characteristics of members of *Enterobacteriaceae* (Bruckner & Colonna, 1997). Hence, to further differentiate these isolates, a selective medium, CHROMagar Orientation™ which yielded different colour morphotypes according to the bacterial genera was used to further differentiate the colony colour morphology of these isolates. These 45 isolates when streaked on the selective medium produced metallic blue colonies after 24 h incubation. Based on the colony colour of these isolates on the CHROMagar Orientation™ medium, they could be either from genus of *Enterobacter*, *Klebsiella* or *Citrobacter* (Merlino et al., 1996; Scarparo et al., 2002). Thus, molecular identification technique was carried out in order to further validate some of these isolated bacteria by sequencing the universal 16S rRNA genes of the bacteria.

4.1.3 Identification of bacterial isolates using molecular techniques

The 15 isolates which fulfilled the characteristics of putative *R. solanacearum,* tested with PCR using primers (OLI-1/Y2) targeting the species-specific 16S rRNA region of the *Ralstonia* sp. produced no desired band (the ~288 bp). This result indicated that none of these 15 isolates could be confirmed as *Ralstonia* sp..

Hence, these unknown species, together with a few representative bacterial isolates with different biochemical characteristics were selected and subjected to PCR amplification using the universal 16S rRNA primers (27f/1492r) which targeted the 16S rRNA genes of the bacteria (produced 1500 bp bands). This latter PCR amplification was carried out

in order to identify all the unknown bacterial isolates. Amplified PCR products (Figure 4.7) were further validated by sequencing technique and Table 4.3 shows the results analysed using the NCBI BLAST System.

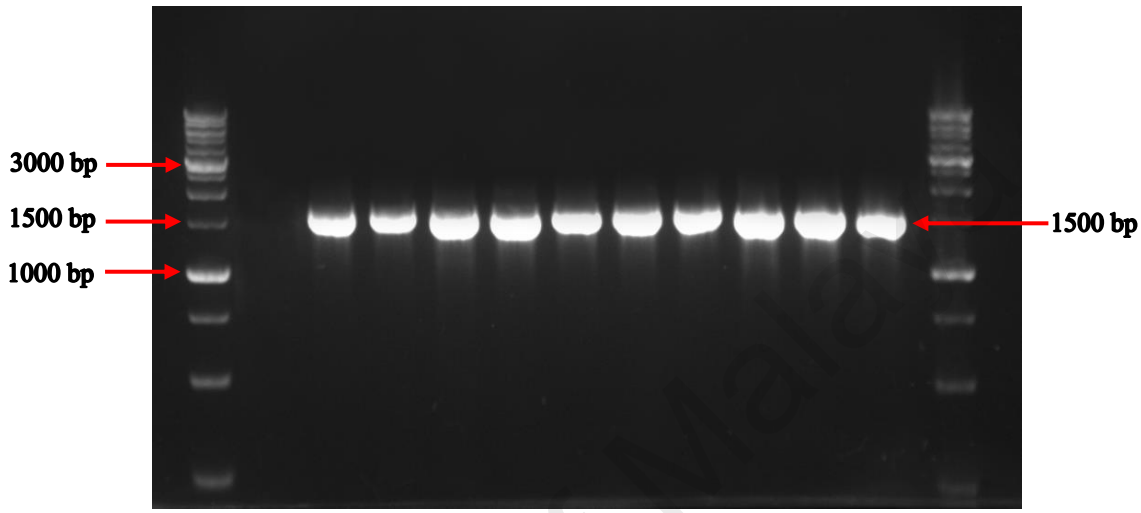

Figure 4.7: The representative gel electrophoresis of PCR amplification with 27f/1492r primers showing 1500 bp bands of 16S rRNA genes of bacterial isolates.

1st Lane (M), 1 Kb molecular size markers; 2, -ve control; 3-12, representative bacterial isolates.

Table 4.3: Sequencing results of representative bacterial isolates targeting 16S rRNA gene

Isolates Designation	Identification	Similarities (%)	Reference sequence (Accession No.)
BP-LN-F1	*Klebsiella pneumoniae*	97.9	NR_074913.1
BP-NI-ST1	*Stenotrophomonas maltophilia*	99.4	NR_041577.1
BP-NI-SL3	*Enterobacter mori*	99.5	NR_116430.1
TC-MS-ST1	*Kosakonia* sp.	99.7	NR_117704.1
TC-MS-SL2	*Enterobacter cloacae*	99.8	NR_118011.1
BT-BR-ST3	*Pseudomonas plecoglossicida*	100	NR_114226.1
BT-BR-O1	*Enterobacter hormaechei*	98.9	NR_042154.1
KP-NA-RT2	*Pseudomonas monteili*	98.9	NR_121767.1
KP-RL-O2	*Klebsiella pneumoniae*	98.4	NR_074913.1
KG-NI-ST2	*Pseudomonas guezennei*	99.2	NR_114957.1

The sequencing results showed that these Gram-negative, catalase and oxidase positive, arginine dihydrolase negative, and non-starch hydrolytic bacteria were identified as either *Pseudomonas* or *Stenotrophomonas.* The Gram-negative, catalase positive, oxidase and arginine dihydrolase negative, with inability to hydrolyse starch bacteria and also produce metallic blue-colonies morphotypes on the CHROMagar Orientation^{TM} medium were either members of *Enterobacter* or *Klebsiella*. Some of these bacteria were also identified as genus of *Kosakonia.* This newly classified genus was previously known as *Enterobacter* (Brady et al., 2013).

In summary, although the rapid detection techniques (ImmunoStrip test and direct PCR amplification) showed presence of *R. solanacearum*, the conventional method failed to isolate the targeted bacterium. Based on the 16S rRNA sequencing results, majority of the isolated bacteria were identified as a few members of *Enterobacteriaceae* (*Enterobacter, Klebsiella, Kosakonia*). Subsequent isolation technique using the non-selective medium was carried out from the samples of symptomatic and non-symptomatic BW-diseased banana plants. The non-selective, Casamino peptone glucose (CPG) agar was used as the isolation medium in order to obtain more diverse bacterial communities from the sampled plants.

4.2 Biofilm-forming *Enterobacteriaceae* isolated from symptomatic and non-symptomatic bacterial wilt-diseased banana plants

4.2.1 Identified bacterial colonies

Sampling was again conducted at the banana orchard in Kampung Sungai Burong, Kuala Selangor, Selangor. Majority of the plants were producing symptoms of BW disease such as yellowing of the leaves and collapse of the petioles forming a skirt of dead leaves around the pseudostem. Furthermore, the field observations also identified that the non-symptomatic young banana plants may suddenly become diseased at the matured stage due to the latent infection. The fruits of these plants appeared normal and unaffected externally, but the pulp displayed brownish black discolouration and rot (Figure 4.8).

Hence, both symptomatic and non-symptomatic banana plants (*Musa paradisiaca* cv. Nipah) were selected from the same infected orchard. The ImmunoStrip test of bacterial ooze samples from both plant samples showed positive results of presence of *R. solanacearum* (as shown in Figure 4.3).

Figure 4.8: Fruit samples from matured infected banana plant. Banana fruits appeared normal from external observation but cross-sections of few banana fingers revealed brownish black discolouration of the pulp (scale bar, 3 cm).

The morphology of the bacterial colonies on the isolation medium, CPG agar were slightly different between the two sampled plants. The non-symptomatic plant sample produced much smaller and less fluidal bacterial colonies, while the colonies isolated from symptomatic plant sample appeared irregular, large and more fluidal (Figure 4.9). Bacterial colonies were carefully picked and further re-streaked onto a fresh CPG agar until pure isolates were obtained (Figure 4.10).

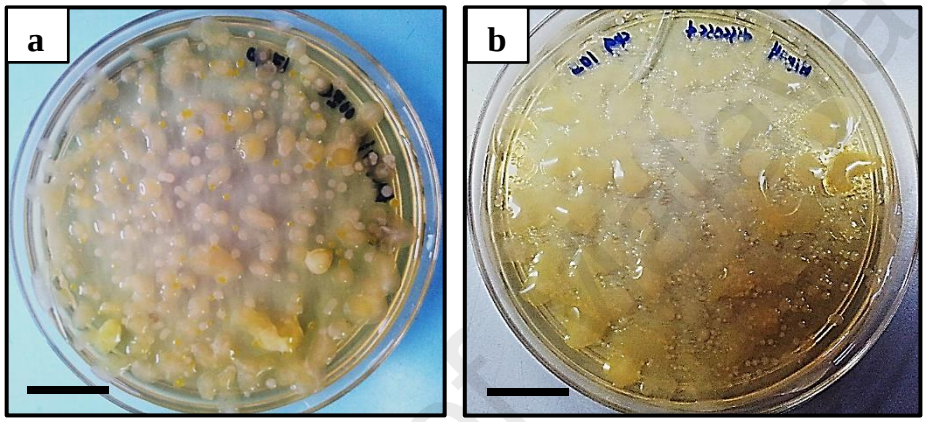

Figure 4.9: Isolated bacterial colonies on the CPG agar from a) non-symptomatic and b) symptomatic plant samples (scale bar, 2 cm).

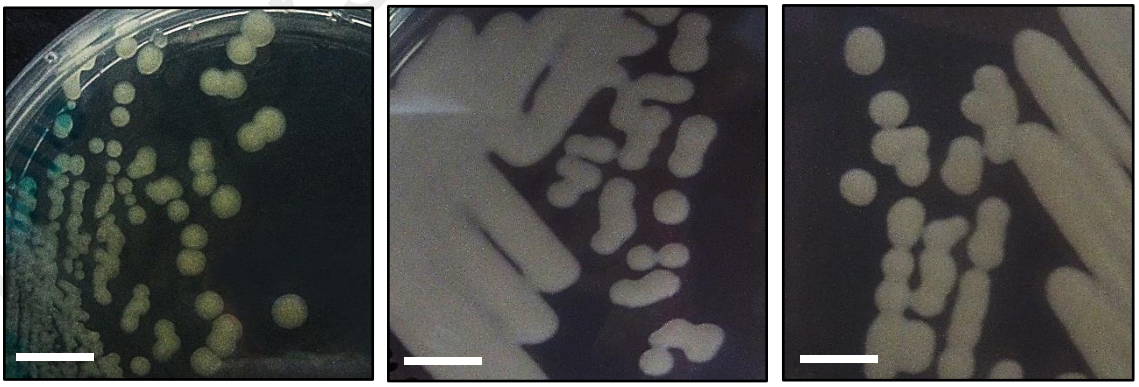

Figure 4.10: Colony phenotypes of different purified bacterial colonies on the CPG agar (scale bar, 1 cm).

All the purified isolates were subjected to 3% (w/v) potassium hydroxide (KOH) string test and those Gram-positive bacteria were eliminated from further identification. Only 28 and 31 Gram-negative bacterial isolates from the non-symptomatic and the symptomatic plants, respectively were further differentiated on the CHROMagar Orientation™ to be grouped into different colony colour morphology. Five different colony colour morphotypes were identified from this medium (Table 4.4).

Table 4.4: Summary of colony colour morphotypes on the CHROMagar Orientation™

Colony colour	**No. of bacterial isolates**	
	Non-Symptomatic Plant	**Symptomatic Plant**
Metallic blue	18	20
Cream	2	4
Dark blue	5	3
Translucent yellow	3	-
Dark maroon	-	4
Total isolates	**28**	**31**

Based on the bacterial colonies colour morphotypes on the CHROMagar Orientation™, representative isolates from each colour morphotypes (from both plant samples) were further selected for molecular identification using the 16S rRNA sequencing technique. Sequencing results for the non-symptomatic and symptomatic plant samples are summarized in Table 4.5 and 4.6, respectively.

Table 4.5: Bacterial Identification of the non-symptomatic plant sample based on CHROMagar Orientation and 16S rRNA sequencing

Bacterial Isolates	CHROMagar Orientation™	Identification	Similarities (%)	Reference sequence (Accession No.)
NS-ST-1	Metallic blue	*Enterobacter sp.*	99.0	NR_118568.1
NS-ST-6		*Enterobacter hormaechei*	100	NR_042154.1
NS-ST-9		*Klebsiella variicola*	100	NR_025635.1
NS-ST-13		*Kosakonia sp.*	100	NR_117704.1
NS-FR-15		*Klebsiella pneumoniae*	99.0	NR_074913.1
NS-FR-21		*Enterobacter sp.*	99.1	NR_118568.1
NS-OZ-26		*Enterobacter hormaechei*	98.4	NR_042154.1
NS-ST-3	Dark blue	*Stenotrophomonas maltophilia*	97.6	NR_074875.1
NS-FR-17		*Stenotrophomonas sp.*	98.9	NR_074875.1
NS-OZ-25		*Stenotrophomonas sp.*	99.0	NR_074875.1
NS-OZ-28		*Erwinia persicina*	98.6	NR_114078.1
NS-FR-18	Translucent yellow	*Aquitalea magnusonii*	99.8	NR_114200.1
NS-OZ-23		*Aquitalea magnusonii*	99.8	NR_043475.1
NS-ST-2	Cream	*Acinetobacter baumannii*	100	NR_074737.1

Table 4.6: Bacterial Identification of the symptomatic plant sample based on CHROMagar Orientation and 16S rRNA sequencing

Bacterial Isolates	CHROMagar Orientation™	Identification	Similarities (%)	Reference sequence (Accession No.)
SY-ST-1	Metallic blue	*Enterobacter cloacae*	99.7	NR_118011.1
SY-ST-4		*Enterobacter hormaechei*	99.0	NR_042154.1
SY-ST-9		*Klebsiella pneumoniae*	100	NR_074913.1
SY-FR-10		*Kosakonia sp.*	99.8	NR_117704.1
SY-FR-14		*Klebsiella variicola*	100	NR_025635.1
SY-ST-17		*Kosakonia sp.*	99.8	NR_117704.1
SY-FR-19		*Enterobacter cloacae*	99.8	NR_044978.1
SY-OZ-22		*Enterobacter oryziphilus*	99.8	NR_125587.1
SY-OZ-24		*Klebsiella pneumoniae*	99.8	NR_074913.1
SY-OZ-27		*Klebsiella variicola*	100	NR_025635.1
SY-ST-12	Cream	*Pectobacterium carotovorum*	98.6	NR_118227.1
SY-OZ-25		*Chryseobacterium nakagawai*	98.8	NR_126257.1
SY-OZ-29		*Sphingobacterium cladoniae*	99.0	NR_108441.1
SY-ST-3	Dark maroon	*Pseudomonas guezennei*	100	NR_114957.1
SY-OZ-28		*Pseudomonas guezennei*	100	NR_114957.1
SY-FR-21	Dark blue	*Erwinia chrysanthemi*	99.2	NR_117738.2

Based on the 16S rRNA gene sequences, the majority group (metallic blue-colonies) from both plant samples were identified as either *Enterobacter*, *Klebsiella* or *Kosakonia* (members of *Enterobacteriaceae*). The minority group of colonies forming colours other than metallic blue were identified as various different genera (non-*Enterobacteriaceae*). As expected, comparing the semi-selective (TZC and SMSA) media and non-selective (CPG) medium, more diverse bacterial species were able to be isolated from the non-selective type medium. From previous sampling using the TZC and SMSA and the latest,

using the CPG media, *Klebsiella* and *Enterobacter*, which are the two members of *Enterobacteriaceae,* were the most abundantly isolated bacteria from all the banana plant, ooze and soil samples. Hence, as an initial step to understand the association of these bacteria with their host plants, twelve representative *Enterobacteriaceae* members (*Klebsiella* sp., *Enterobacter* sp., and *Kosakonia* sp.) were selected for further characterization on the biofilm-forming ability.

4.2.2 Representative bacterial strains showed the ability to form biofilm

The *in-vitro* biofilm-forming ability of 12 bacterial strains were examined. All these representative bacteria showed the ability to form biofilm based on the microtiter plate assay test. The amount of biofilms produced by these bacterial strains were determined by measuring the intensity of crystal violet (0.1%) that stained the adhered biofilms, spectrophotometrically (Appendix E).

The optical density (O.D) readings for all the tested bacterial strains in three independent experiments are summarized in Table 4.7. Based on the calculated average of the O.D reading, three strains were moderate biofilm producers ($0.65 < A_{590\ nm} < 1.3$), and nine others were strong biofilm producers (A_{590} nm > 1.3). Four of these strong biofilm producers with highest OD readings were further selected for *in-vivo* assessment.

Table 4.7: Biofilm-forming ability of twelve bacterial strains based on the average O.D reading of three independent experiments

Bacterial Species	Average Optical density (O.D) reading				Biofilm-forming ability**
	1st Experiment	2nd Experiment	3rd Experiment	Average*	
Enterobacter hormaechei	3.89	3.57	4.12	**3.86**	Strong producer
Klebsiella pneumoniae	1.73	1.21	1.29	**1.41**	Strong producer
Klebsiella pneumoniae	2.04	2.18	2.06	**2.09**	Strong producer
Enterobacter cloacae	1.98	2.54	2.38	**2.30**	Strong producer
Klebsiella variicola	1.42	2.3	1.95	**1.89**	Strong producer
Kosakonia sp.	2.64	3.55	3.01	**3.07**	Strong producer
Enterobacter cloacae	1.29	1.46	1.33	**1.36**	Strong producer
Enterobacter oryziphilus	0.74	0.71	0.94	**0.80**	Moderate producer
Klebsiella variicola	1.64	1.29	1.28	**1.40**	Strong producer
Klebsiella pneumoniae	0.99	0.87	0.92	**0.93**	Moderate producer
Enterobacter hormaechei	1.13	2.05	2.12	**1.77**	Strong producer
Kosakonia sp.	1.16	1.24	1.17	**1.19**	Moderate producer

*Moderate biofilm producers ($0.65 < A_{590\ nm} < 1.3$) and strong biofilm producers ($A_{590\ nm} > 1.3$).

**Average for the three independent experiments.

4.3 Co-inoculation of biofilm-forming bacteria with the blood disease pathogenic strain, *Ralstonia syzygii* subspecies *celebesensis* into the banana plants in glasshouse conditions

In this glasshouse experiment, the four biofilm-forming strains were inoculated as mono- and co-culture with the pathogenic strain, *Ralstonia syzygii* subspecies *celebesensis* into the two months old banana plants according to their assigned treatments (Table 3.3). The severity of infections caused by those inoculated bacteria, bacterial colonization and EPS production in vascular system of the infected and non-infected host plants were observed.

4.3.1 Observation on the external and internal symptoms of the inoculated plants

After a few days of inoculation, some inoculated banana plants produced unusual patches of decolourization on the leaves surfaces around the inoculation site (Figure 4.11).

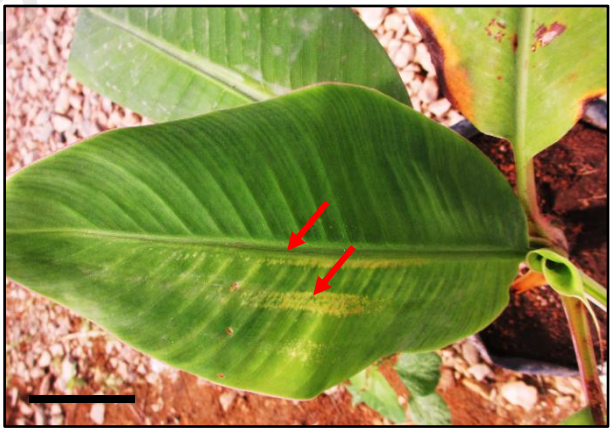

Figure 4.11: Decolourized patches on the leaf surface observed around the inoculation site after five days of treatment. Red arrows indicate the observation (scale bar, 3 cm).

On the third week of post-inoculation (3rd WPI), two plants from Treatment 1 (co-inoculated with *Klebsiella pneumoniae* and BDB) and one from the positive control treatment (T9) started to show clear wilting symptoms (Figures 4.12a and 4.12b).

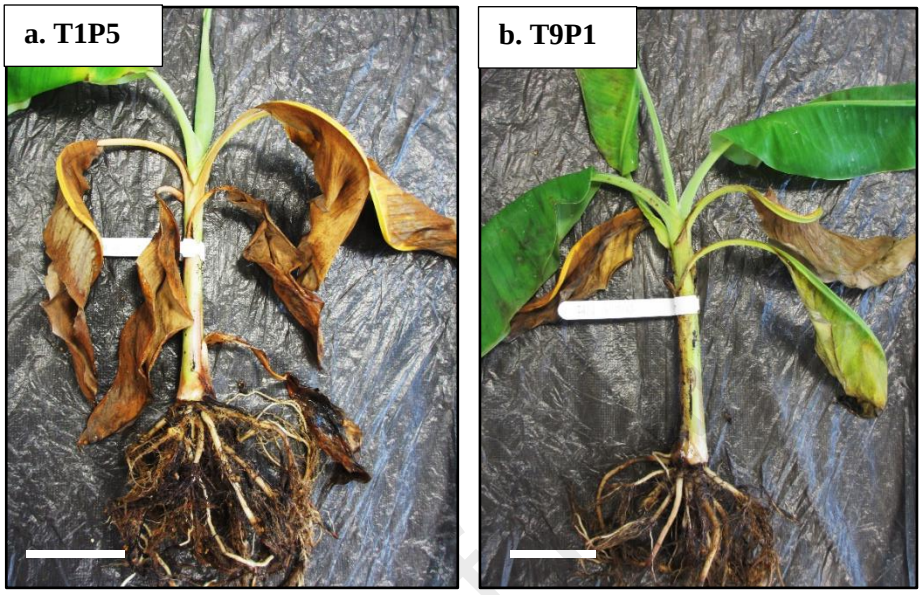

Figure 4.12: Some individual plants from a) Treatment 1 (T1P5) and b) Treatment 9 (T9P1) showed positive wilting symptoms (scale bar, 4 cm).

In other treatments, some plants showed unusual signs such as uncommon growth and stunted, crooked and burned young leaves, and also non-typical (fan-shaped) leaves arrangement. Those plants were either treated with biofilm-forming bacteria (BFB) only or those combined with BDB (*Ralstonia syzygii* subsp. *celebesensis*) (Figures 4.13a – 4.13c).

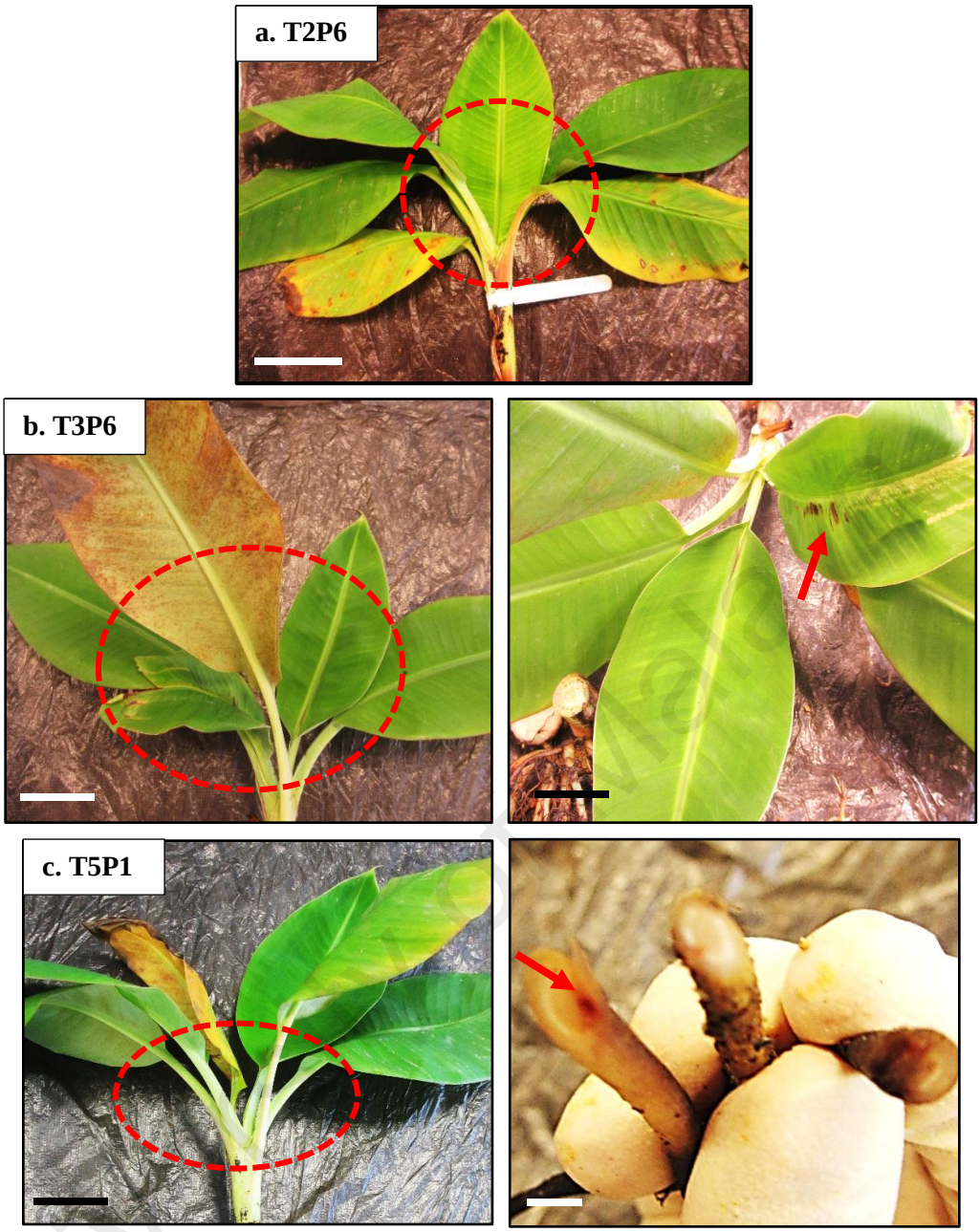

Figure 4.13: Representatives of inoculated plants showing the unusual symptoms a) Uncommon growth of young leaf in T2P6, b) Stunted, crooked and burned young leaf with non-typical (fan-shaped) leaves arrangement of the T3P6 plant, c) Fan-shaped leaves arrangement and reddish stripes in some roots of T5P1. Red dotted circles and arrows indicate the symptoms (scale bar, a and b =4 cm, c =3 cm and 1 cm).

Until the fourth week (4th WPI), not all plants from the positive control (T9) and those inoculated with BDB and BFB strains (T1, T3, T5, and T7) showed diseased symptoms. A few T1, T5 (co-inoculated with *Kosakonia radicincitans* and BDB) (Figure 4.14) and

T9 plants produced wilting and epinasty symptoms. During this week (4th WPI), not a single plant from T3 (co-inoculated with *Enterobacter cloacae* and BDB) and T7 (co-inoculated with *Enterobacter hormaechei* and BDB) indicated any symptoms of the disease.

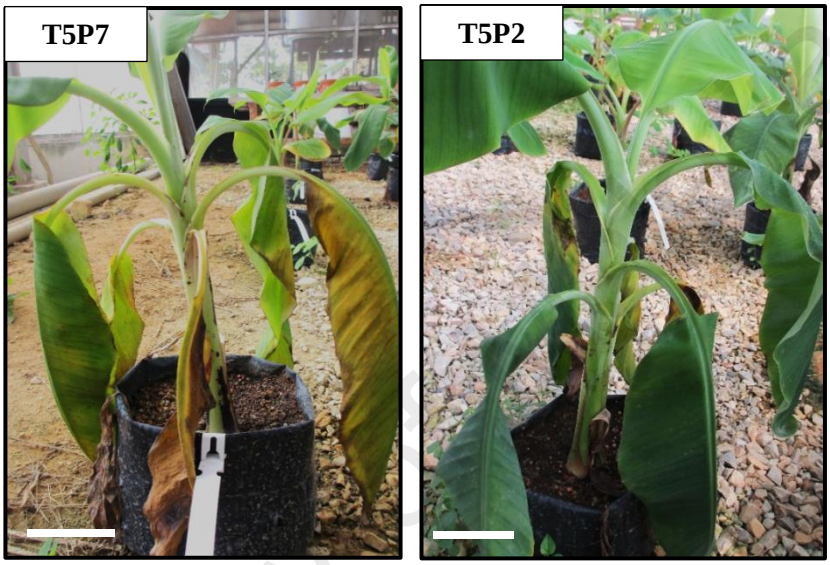

Figure 4.14: Two representative plants from T5 (co-inoculated with *Kosakonia radicincitans* and BDB) with symptoms of leaves epinasty (T5P2) and light wilt (T5P7) at the 4th week of observation (scale bar, 6 cm and 7 cm).

During the 5th week observation (5th WPI), more plants from T1 produced wilt symptoms and those plants showing symptoms from T5 and T9 were more severely wilted. During this week, two plants from T7 started to have some external or internal wilt symptoms (Figures 4.15a and 4.15b). However, all T3 plants were still free from any diseased symptoms till the 5th WPI.

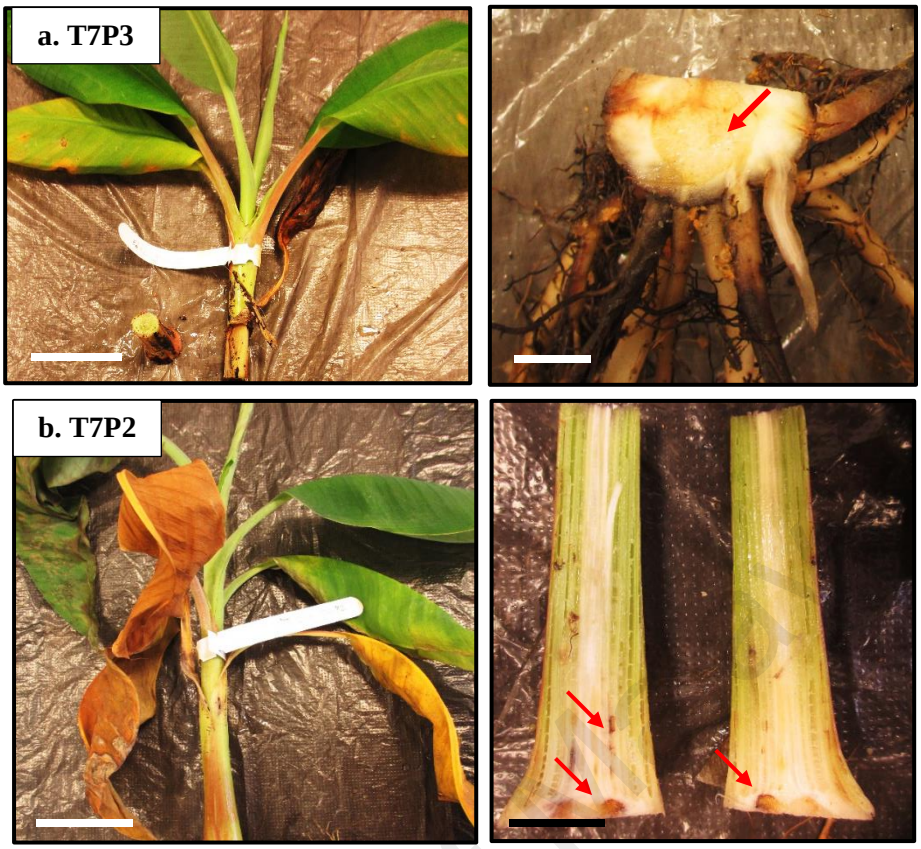

Figure 4.15: Two representative plants from T7 (co-inoculated with *Enterobacter hormaechei* and BDB) at the 5th week of observation, a) T7P3 plant was normal from the external observation but the rhizome of the plant showed browning of the tissues and some roots were rotten, b) T7P2 plant showed wilting symptoms from the outside and cross-sectioned of the pseudostem and rhizome exposed the browning of vascular bundles. Red arrows indicate the symptoms (scale bar, a =3 and 1 cm, b =3 and 2 cm)

Those plants from treatments inoculated with only BFB strains were disease-free yet some of the plants produced unusual symptoms of stunted growth, scorched leaves (Figure 4.16) or non-typical (fan-shaped) leaves arrangement.

Only at the 6th week of data collection, all treatments with the presence of BDB have at least one plant up to five plants per treatment which clearly indicated bacterial wilt symptoms. Some of those inoculated plants were severely wilted and eventually died (Figures 4.17a – 4.17c).

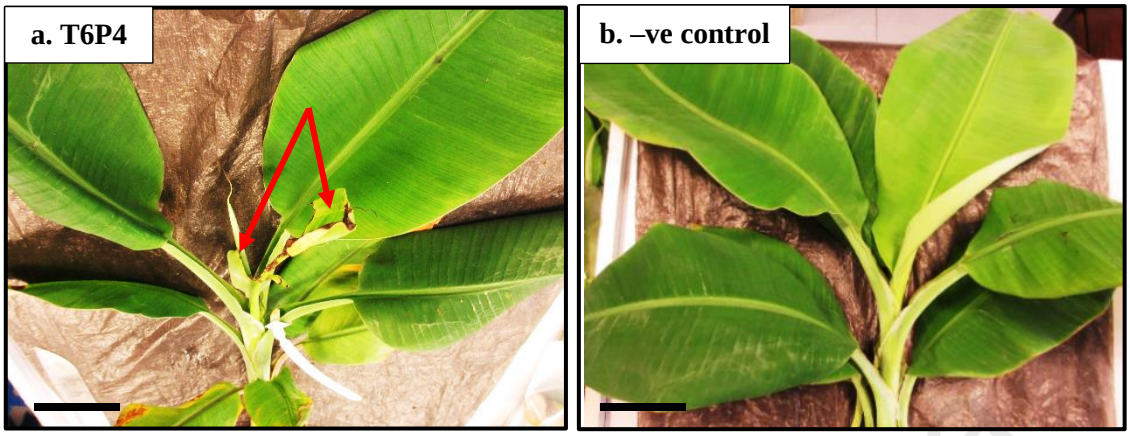

Figure 4.16: Unusual symptoms of a plant inoculated with only biofilm-forming bacterium (BFB), a) T6P4 plant showed burnt young shoot around the inoculation site (indicated by red arrows), b) typical growth of negative control plant (scale bar, 4 cm)

Figure 4.17: Representative plants from different treatments with pathogenic strain, BDB showing clear bacterial wilt symptoms, a) T1P6 plant with rhizome and pseudostem producing brown stripes (red arrows indicate the symptoms), b) T5P2 plant was totally wilt and the cross-sectioned showed the rotten rhizome, c) positive control plant (inoculated with BDB) showed severe symptom (scale bar, a =9, 3 and 1 cm, b =8 and 2 cm, c =5 and 2 cm)

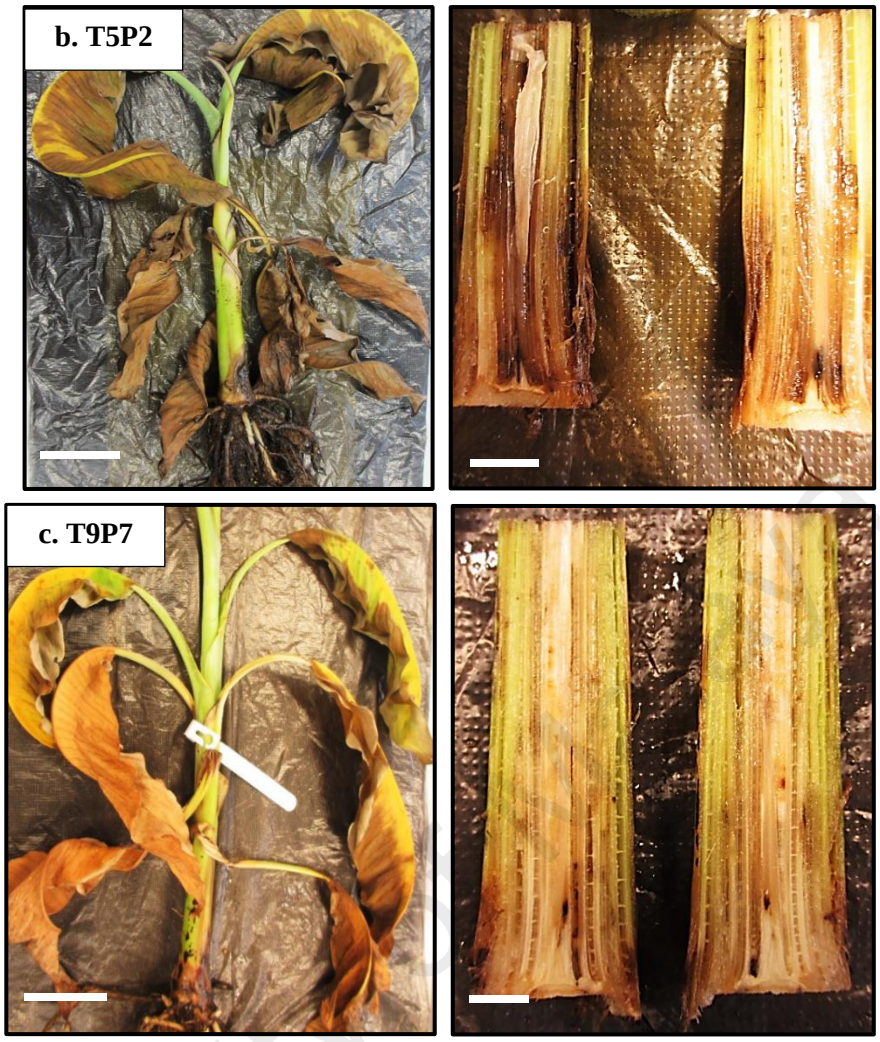

Figure 4.17, continued.

Another interesting observation on the 6th week was the different physical appearances of a few plants from different treatments (Figure 4.18). Apparently, dissimilarity of the external conditions of four individual plants from four different treatments were evident. All seven plants from Treatment 8 (inoculated with *Enterobacter hormaechei*) were healthy and disease-free. The physical observation indicated that three T8 plants were obviously healthier than other plants in all treatments including those from the negative control. Comparison of plants in Treatment 5 (inoculated with BDB and *Kosakonia radicincitans*) and positive control plants (T9) showed that T5 plants were more severely affected by the presence of pathogenic agent as compared to T9, although the volume of

Ralstonia syzygii subsp. *celebesensis* strain (BDB) used as inoculant in T5 plants were lesser (2.5 ml of BDB + 2.5 ml of *K. radicincitans*) compared to the volume in T9 (5 ml of BDB).

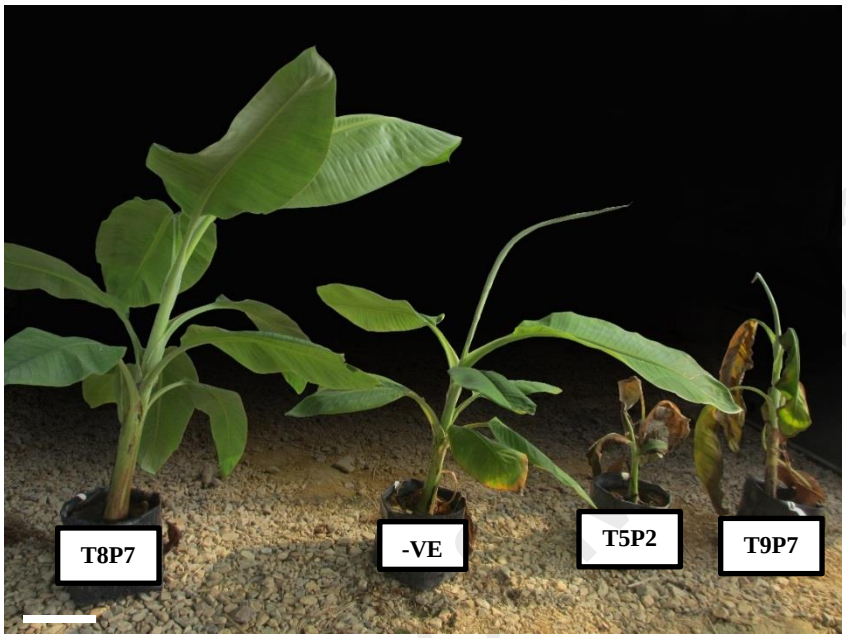

Figure 4.18: Comparison of four plants from four treatments with clear distinct physical appearances (scale bar, 4 cm)
From the left: A plant from Treatment 8 (T8P7) was vigour and very healthy, a negative control (-VE), a plant from Treatment 5 (T5P2) was the most severely wilted and dying, while a positive control (T9P7) was also wilted but less severe than that of T5.

Furthermore, based on the weekly recorded ESI and ISI scales of all the inoculated plants, the disease symptoms developed more rapidly in the plants under the two treatments (T1 and T5) as compared to those plants under the positive control treatment (T9) (Figure 4.19).

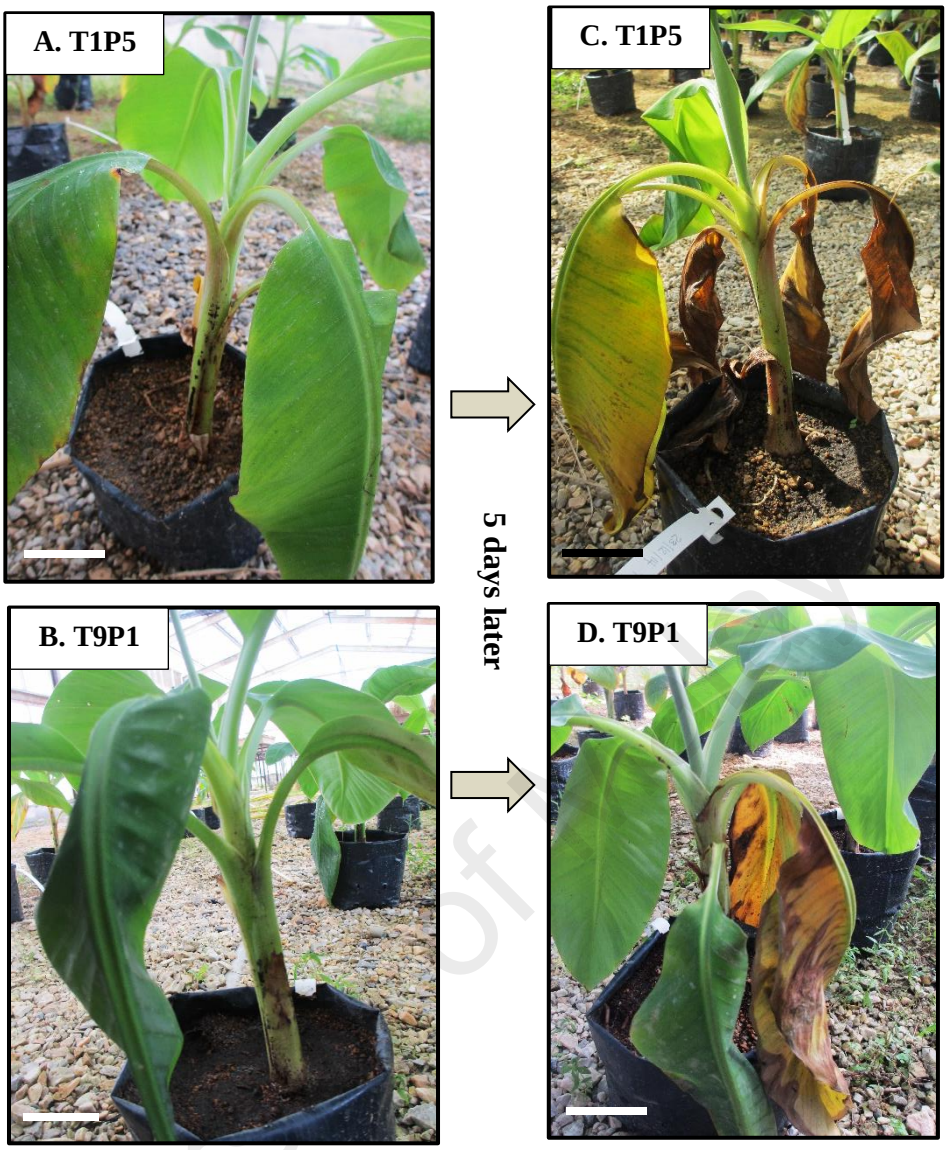

Figure 4.19: Comparison of a plant from A) Treatment 1 (T1P5) and B) Treatment 9 (T9P1) which developed a mild symptom at the 4th WPI. After 5 days, the same C) T1P5 plant was showing severely wilt symptoms as compared to D) T9P1 plant with lesser severity of infection (scale bar, A and C =7 cm, B and D =8 cm)

4.3.2 Interpretation of disease severity

Based on the observations, the external symptoms index (ESI) and internal symptoms index (ISI) scores were recorded weekly (Appendix F). For each given ESI and ISI scales for every treatment, disease severity index (DSI) was further calculated and interpreted based on the formula described in sub-chapter 3.3.3 (Appendix G). The final status of

disease expression for plants under each treatment were given based on the total interpretation of DSI scores from both ESI and ISI scales (Table 4.8).

Table 4.8: Response of banana plants to the inoculated bacterial suspensions at the eighth weeks of post inoculation (8^{th} WPI).

Treatment	Bacterial suspension*	No. of infected plants**	DSI Scores***		Final Status of the Disease Expression
T1	2.5 ml of KP + 2.5 ml of BDB	5	ESI	3.7	Highly susceptible
			ISI	2.7	
T2	5 ml of KP	0	ESI	1.7	Tolerant
			ISI	1.3	
T3	2.5 ml of EC + 2.5 ml of BDB	2	ESI	2.4	Susceptible
			ISI	1.9	
T4	5 ml of EC	0	ESI	1.7	Tolerant
			ISI	1.0	
T5	2.5 ml of KR + 2.5 ml of BDB	6	ESI	4.0	Highly susceptible
			ISI	3.3	
T6	5 ml of KR	0	ESI	1.6	Tolerant
			ISI	1.0	
T7	2.5 ml of EH + 2.5 ml of BDB	4	ESI	2.7	Susceptible
			ISI	2.3	
T8	5 ml of EH	0	ESI	1.0	Resistant
			ISI	1.0	
T9 (+VE)	5 ml of BDB	6	ESI	3.8	Highly susceptible
			ISI	3.0	
-VE	5 ml CPG broth	0	ESI	1.0	Resistant
			ISI	1.0	

*KP = *Klebsiella pneumoniae,* EC = *Enterobacter cloacae,* KR = *Kosakonia radicincitans,* EH = *Enterobacter hormaechei,* BDB = *Ralstonia syzygii* subsp. *celebesensis*, CPG = Casamino peptone glucose. The concentration of bacterial suspension used equals to 1.0 x 10^7 cfu/ml (this is equivalent to optical density at 600 nm, 0.3).

**Infected plant is defined as plant with ESI and ISI scales of 3 to 5.

***ESI = External Symptom Index and ISI = Internal Symptom Index. The disease severity index (DSI) for each ESI and ISI scores was calculated and interpreted as resistant, tolerant, susceptible and highly susceptible. The final status of disease expression for each treatment depends on the more severe result of interpretation of DSI scores (Mak et al., 2004).

For all the treatments, the DSI interpretations for both ESI and ISI scales were either the same or the ESI scales were interpreted as more severely infected than the ISI scales. Plants that were under co-inoculation (T1, T3, T5, and T7) and positive control (T9) treatments were either susceptible or highly susceptible to the disease. Plants that were subjected to monoculture inoculation treatments (T2, T4, and T6) were tolerant to the disease. Noteworthy, only plants under T8 were interpreted as resistant (as recorded for plants under the negative control). Out of all the treatments including the positive control (T9), the DSI for both ESI and ISI scales for T5 were interpreted as highly susceptible. This result indicated that plants under T5 were the most severely affected and infection was progressing rapidly throughout the plant. On the other hand, the ISI score (based on internal symptoms) for T3 was interpreted as tolerant, even though the pathogenic strain (BDB) was co-inoculated into the plants.

Statistical analysis based on the independent-samples Kruskal Wallis test (the significance level, $p < 0.05$) depicted that there are significant differences across all treatments for the ESI and ISI scales. However, for the ESI scales, only T2, T4, T6 and T8 (treatments with mono-inoculation of only biofilm-forming strains) are significantly different from the T9 (positive control). There are no significant difference between T1, T3, T5 and T7 (treatments with co-inoculation of biofilm-forming strains and pathogenic strain) with the T9. Meanwhile, for the ISI scales, only T4, T6 and T8 are significantly different from the T9, and T1, T2, T3, T5 and T7 have no significant differences with T9 (Appendix G). At the end of the observation period, fragments of the rhizomes tissues of representative plants from each treatment were further examined under the SEM to determine the colonization conditions of inoculated bacteria in their host plants.

4.3.3 Identification of re-isolated bacteria from the inoculated banana plants with and without blood disease symptoms

Re-isolation was done from the inoculated plants from each of the treatment (Figure 4.20). Plants were expected to be colonized by those inoculated bacterial strains. Plants under treatments with co-inoculation of *Ralstonia syzygii* subsp. *celebesensis* (BDB) and BFB (T1, T3, T5 and T7) mostly produced clear wilting symptoms. In contrast, plants under mono-culture inoculation treatments (T2, T4, T6 and T8) did not show any wilting symptoms. Nevertheless, inoculated biofilm-forming bacteria could be re-isolated out (Figure 4.21).

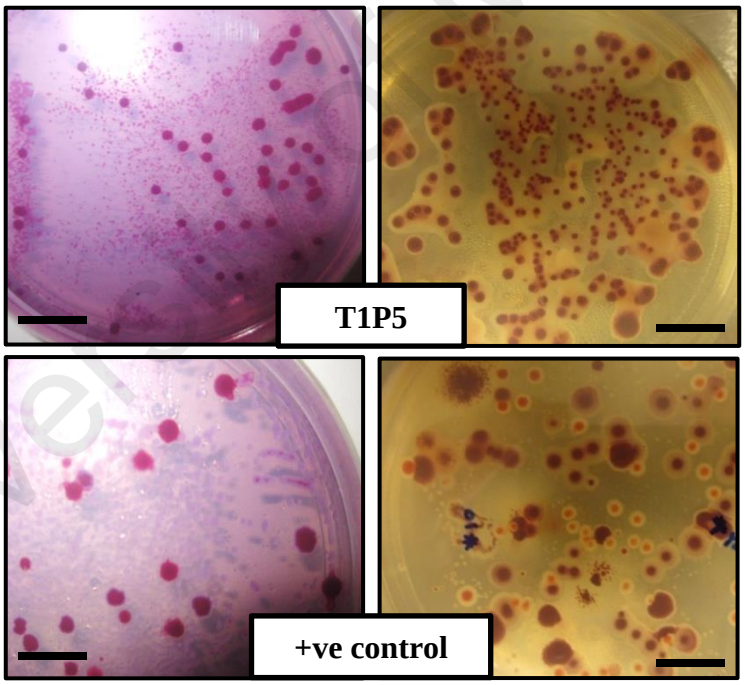

Figure 4.20: Some of the bacterial colonies on the SMSA and TZC media, isolated from the infected banana plants. On the SMSA medium, majority of bacterial isolates were BDB-like colonies (scale bar, 2 cm)

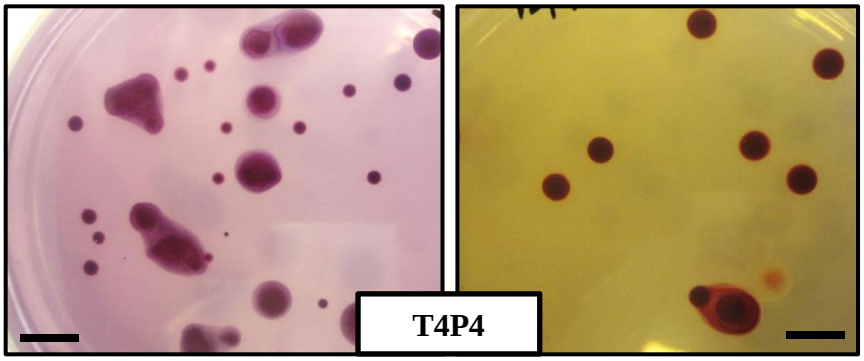

Figure 4.21: Bacterial colonies isolated from non-infected banana plant on TZC and SMSA media. Plants under Treatment 4 (T4P4) were inoculated only with a BFB, *Enterobacter cloacae* (scale bar, 2 cm)

Putative BDB isolates were further purified and identified using the Polymerase Chain Reaction (PCR) technique with *R. solanacearum* species complex primers, 759/760 which produced ~282-bp bands for BDB strains (Figure 4.22). Following that, confirmation was done through the amplification and sequencing of partial 16S rRNA genes of the isolated bacteria. As expected, all the inoculated bacterial strains (BDB and BFB) could be re-isolated from plants under each treatment at the eighth weeks of post inoculation **(**8th WPI) (Table 4.9). This implies that the bacteria could be re-isolated from the experimentally inoculated host and identified as being identical to the original one (Koch's postulates fourth criteria).

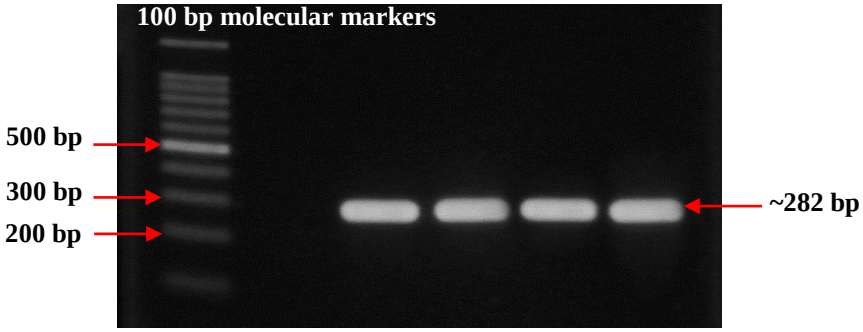

Figure 4.22: Amplicons of putative BDB isolates (~282-bp bands) after the amplification with *R. solanacearum* species complex primers, 759/760

Table 4.9: Identification of bacterial strain re-isolated from selected plants under each treatment at the eighth weeks of post inoculation **(**8th WPI).

Treatment	Identification	Similarities* (%)	Reference sequences (Accession No.)
T1	*Klebsiella pneumoniae subsp. pneumoniae*	100	NR_074913.1
	Ralstonia syzygii	Produced 282-bp fragment**	
T2	*Klebsiella pneumoniae subsp. pneumoniae*	99.4	NR_074913.1
T3	*Enterobacter cloacae subsp. dissolvens*	99.4	NR_118011.1
	Ralstonia syzygii	Produced 282-bp fragment	
T4	*Enterobacter cloacae subsp. dissolvens*	100	NR_118011.1
T5	*Kosakonia radicincitans*	99.7	NR_117704.1
	Ralstonia syzygii	Produced 282-bp fragment	
T6	*Kosakonia radicincitans*	99.5	NR_117704.1
T7	*Enterobacter hormaechei*	98.5	NR_042154.1
	Ralstonia syzygii	Produced 282-bp fragment	
T8	*Enterobacter hormaechei*	100	NR_042154.1
+ve control (T9)	*Ralstonia syzygii subsp. celebesensis*	99.6	NR_025975.1

*[1]DNA amplified with 16S rRNA sequencing and the results were analysed using the NCBI BLAST System (http://www.blast.ncbi.nlm.nih.gov/ BLAST).

**~282-bp fragments produced using Polymerase Chain Reaction technique with *Ralstonia* species-complex primers, 759/760 (Villa et al., 2003).

4.3.4 Scanning electron microscope images showed structural changes in plant tissues and colonization patterns of the inoculated bacteria

At the end of data collection week in the glasshouse (at the 8th WPI), a plant from the negative and positive controls (T9) and from all the treatments (T1-T8) were chosen for the scanning electron microscopic observation. Fragments of tissues from plant rhizome parts (Figures 4.23a and 4.23b) were observed with different magnification scales.

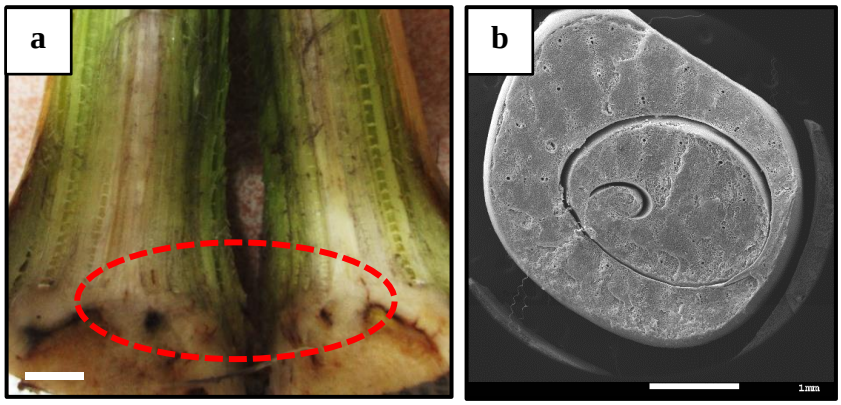

Figure 4.23: Bacterial colonization observed in fragments of pseudostem tissues a) Dotted circle showing the area of tissue fragments chosen for the observation under the scanning electron microscope (SEM) and b) fragments of middle area of pseudostem tissues observed under the SEM (scale bar, a =2 cm and b =1 mm)

Negative control plant did not show any unusual symptoms from the external observation. SEM images of the xylem vessels of the plant also showed a turgor cell wall structure without colonization of bacteria (Figure 4.24).

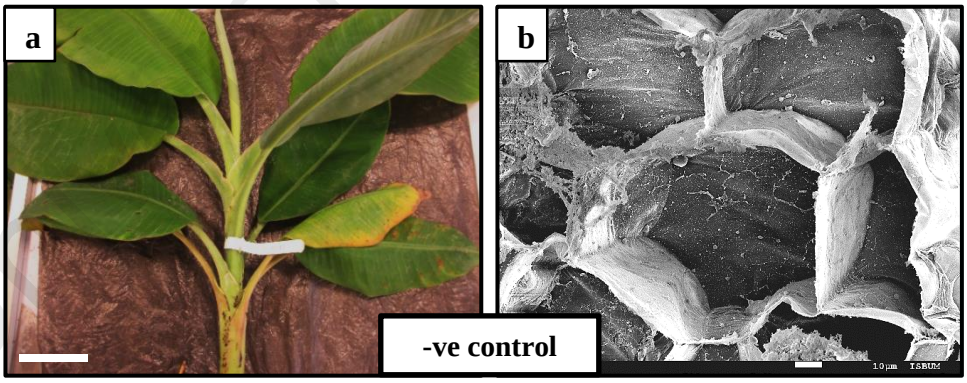

Figure 4.24: Representative of negative control plants (inoculated with CPG broth only), a) The external upper part b) xylem vessels were thick and turgid without any colonization of bacteria (scale bar, a =6 cm, b =100 µM)

4.3.4.1 Observations of the non-infected plant tissues

Plants under treatments inoculated only with BFB did not produce any wilting symptoms. Based on the SEM images, none of the non-wilted plants showed changes in the cell wall structure of the vasculature tissues. Plants from Treatment 2 (Figure 4.25), Treatment 4 (Figure 4.26) and Treatment 8 (Figure 4.27) were free from any bacterial colonization. However, xylem vessels in vasculature tissues of plant from Treatment 6 indicated some cellular structures of bacterial cells (Figure 4.28).

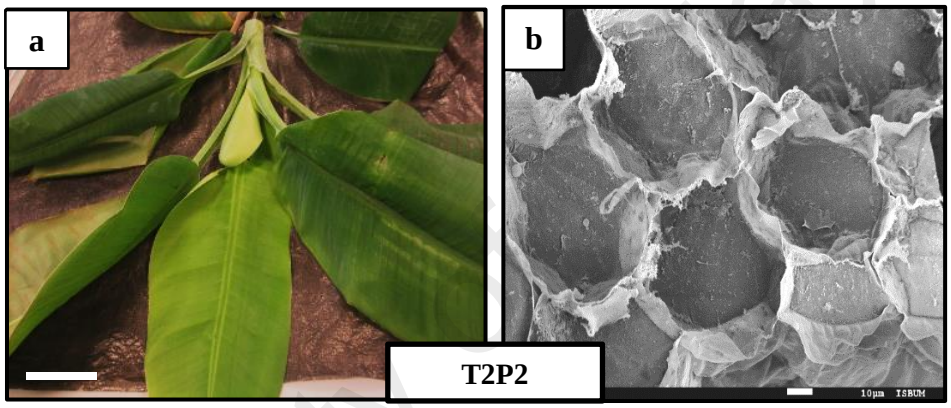

Figure 4.25: Representative of T2 plants (inoculated with BFB, *Klebsiella pneumoniae*) showing no infection, a) The external upper part of T2P2 plant b) vasculature tissues showing no colonization of bacteria (scale bar, a =5 cm, and b =10 µM)

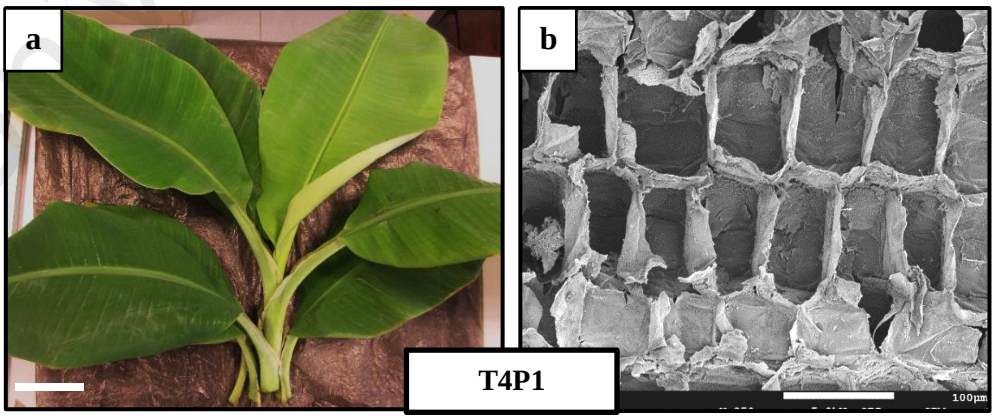

Figure 4.26: Representative of T4 plants (inoculated with BFB, *Enterobacter cloacae*) showing no infection, a) The external upper part of T4P1 plant b) cross-section of xylem tissues showing no colonization of bacteria (scale bar, a =4 cm, and b =100 µM)

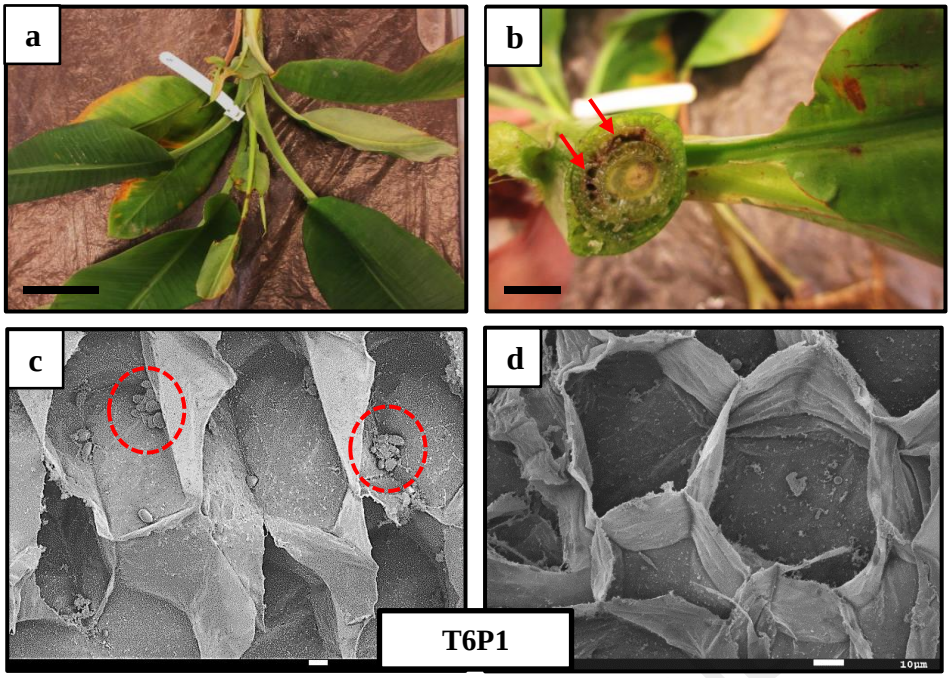

Figure 4.27: Representative of T6 plants (inoculated with BFB, *Kosakonia radicincitans*) showing non-typical external symptoms, a) The external upper part of T6P1 plant b) cross-section of pseudostem part indicated burned tissues at the inoculation site (red arrows) c) cross-section of xylem vessels with a few clumps of bacterial cells (red dotted circle) d) xylem vessels without bacterial aggregates (scale bar, a =6 cm, b =1 cm, c and d =10 µM)

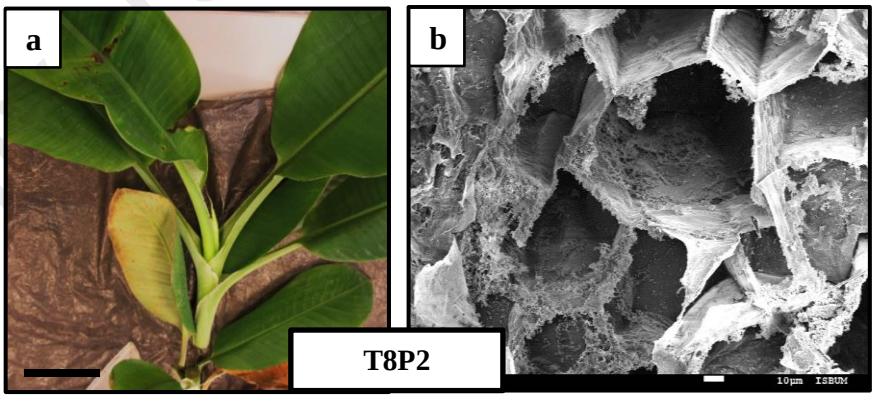

Figure 4.28: Representative of T8 plants (inoculated with BFB, *Enterobacter hormaechei*) showing no infection, a) The external upper part of T8P2 plant b) xylem vessels were turgid without any colonization of bacteria (scale bar, a =7 cm, and b =10 µM)

4.3.4.2 Observations of the infected plant tissues

Symptomatic plant samples were collected from treatments co-inoculated with *Ralstonia syzygii* subsp. *celebesensis* (pathogenic bacteria) and BFB (T1, T3, T5 and T7), and also from the positive control treatment (T9). SEM images of the rhizome tissues of plants from these treatments were captured in order to observe the adhesion and colonization conditions by the inoculated bacteria, accumulations of extracellular polymeric substances (EPS) and structural changes in infected plant tissues.

T1P4 plant from Treatment 1 showed clear wilting symptoms, based on the external and internal symptoms. The plant was totally wilted and died while the internal part of pseudostem and rhizome were discoloured (Figures 4.29a and 4.29b). Based on the SEM images, bacterial aggregates were detected in most of the xylem vessels of plant tissues. The bacterial cells were tightly attached to the xylem wall and appeared interconnected to each other and meet roughly in the centre of the xylem lumen where they fuse (Figure 4.29c). Figure 4.29d reveals the structures which are composed of EPS-encased bacteria, developed from the adherent cells.

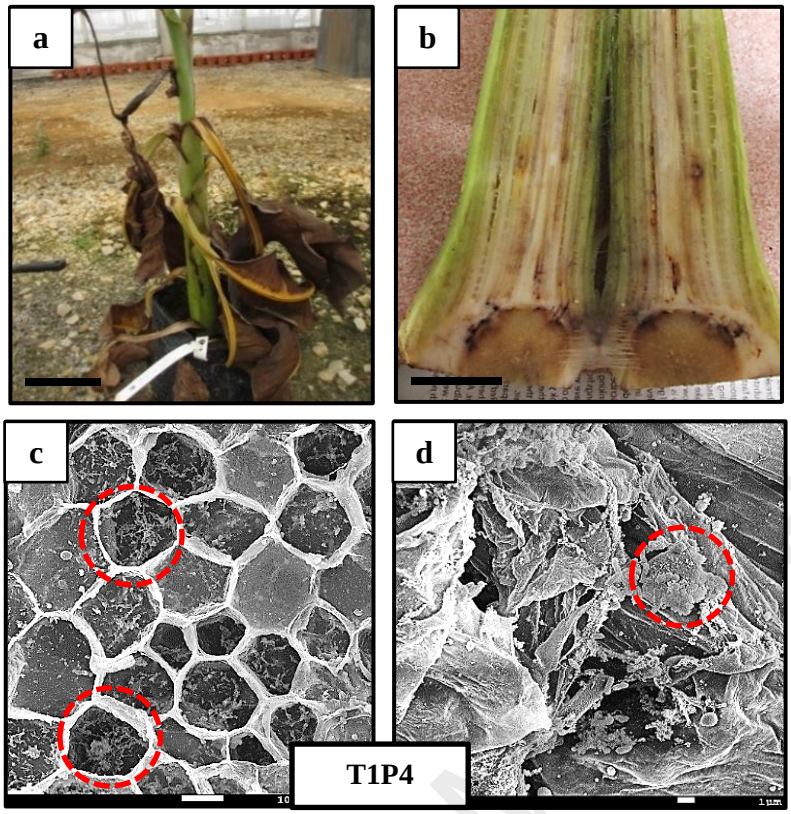

Figure 4.29: Representative of T1 plants (co-inoculated with *Enterobacter hormaechei* and BDB) showing bacterial wilt symptoms, a) The external upper part of T2P4 plant showed total wilt b) cross-section of pseudostem tissues showed total discolouration c) bacterial cells colonizing the xylem vessels d) xylem tissues were collapsed and structures of exopolysaccharides (EPS)-encased bacterial agglomerates adhered to the xylem vessels (scale bar, a =7 cm, b =2 cm, c =10 µM and d =1 µM)

A plant from treatment 3, T3P1 was partly wilted and narrow dark stripes appeared at the vascular bundles of pseudostem (Figures 4.30a and 4.30b). Observation under the SEM revealed less densely populated bacterial cells residing in the vascular tissues and the tracheal elements of xylem vessels (Figures 4.30c and 4.30d). The bacterial aggregates were invariably collapsed to one side of the tracheal wall and lack of EPS fibril material.

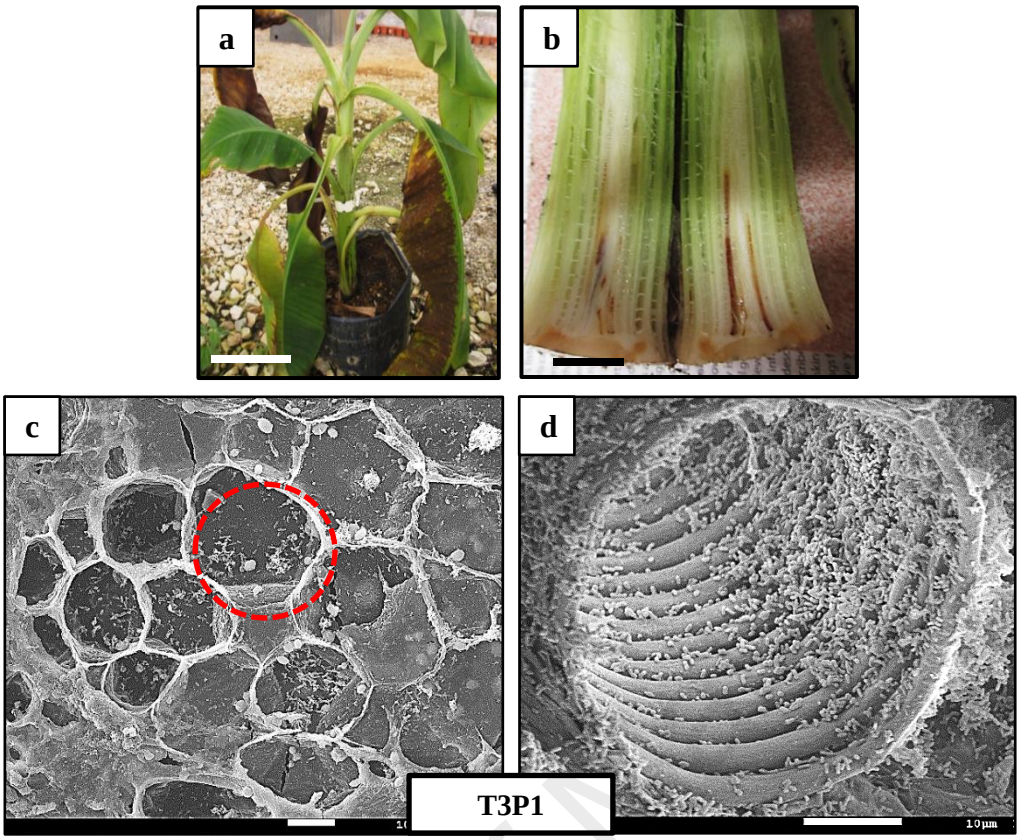

Figure 4.30: Representative of T3 plants (co-inoculated with *Enterobacter cloacae* and BDB) showing bacterial wilt symptoms, a) The external condition of T3P1 plant b) cross-section of pseudostem revealed narrow dark stripes at the vascular bundles c) and d) less densely populated bacteria lacking of EPS fibril material in the xylem vessels (scale bar, a =7 cm, b =2 cm, c and d =10 µM)

Based on the external observation, a plant from treatment 5, T5P3 was severely wilt and dying. The cross-section of pseudostem and rhizome parts revealed total discolouration and flaccid tissues (Figures 4.31a and 4.31b). The SEM images indicated the colonization of bacterial aggregates tightly attached to xylem vessels and adherent cells forming a 3D structures composed of EPS-encased bacteria (Figure 4.31c). Figure 4.31d shows vascular tissues were collapsed and covered by heavy biofilm structures blocking the vessels.

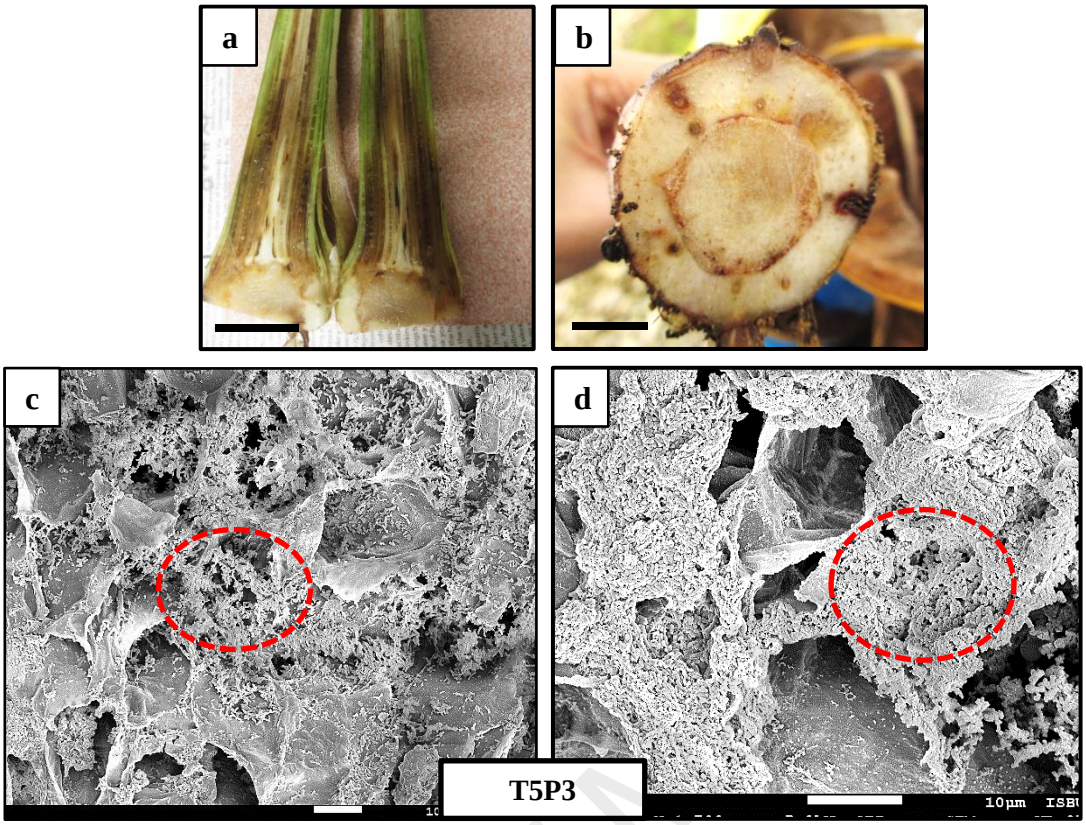

Figure 4.31: Representative of T5 plants (co-inoculated with *Kosakonia radicincitans* and BDB) showing bacterial wilt symptoms, a) the cross-section of discoloured, flaccid pseudostem tissues b) cross-section of rhizome revealing more than 50% discolouration c) bacterial aggregates were tightly attached to the xylem wall and developed a 3D structures composed of EPS-encased bacteria d) heavily EPS-enmeshed bacterial cells blocking the xylem vessels (scale bar, a =2 cm, b =1 cm, c and d =10 µM)

T7P1 plant showed mild wilting symptoms from the external observation (Figure 4.32a). The cross section of plant showed discoloured rhizome tissues (Figure 4.32b). The SEM images revealed a less densely populated bacterial agglomerates which were heavily enmeshed in EPS. However, this structure was invariably collapsed to one side of the xylem wall (Figures 4.32c and 4.32d).

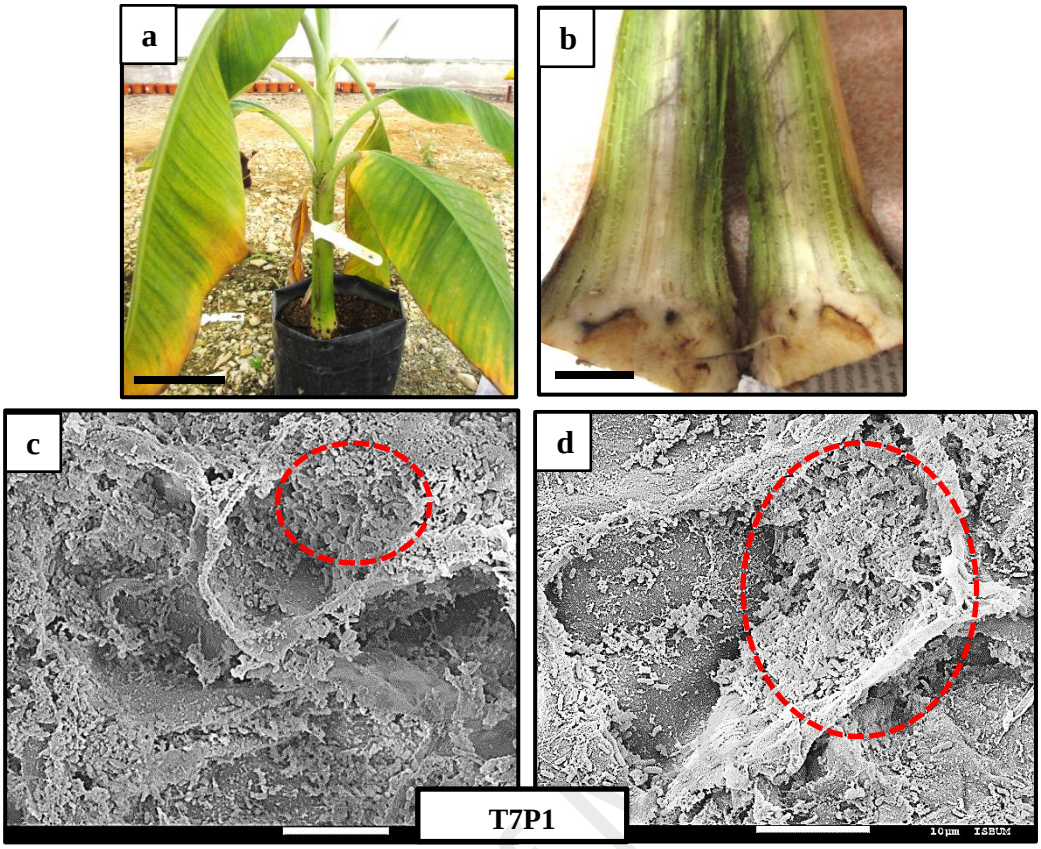

Figure 4.32: Representative of T7 plants (co-inoculated with *Enterobacter hormaechei* and BDB) showing bacterial wilt symptoms, a) half-wilted plant of T7P1 b) The cross-section of plant showed discoloured rhizome tissues c) and d) bacterial aggregates developed into clumps of EPS-encased structure. However, this structure collapsed to one side of the xylem wall (scale bar, a =7 cm, b =2 cm, c and d =10 µM)

T9P4 was severely infected with all leaves wilted and bent down (Figure 4.33a). The internal of pseudostem part was fully discoloured and tissues were rot (Figure 4.33b). The SEM images indicated the accumulation of bacterial clumps with biofilm structures in the xylem vessels causing the xylem walls to rupture (Figure 4.33c). Flocculent materials were indicative of EPS matrix in a biofilm (Figure 4.33d).

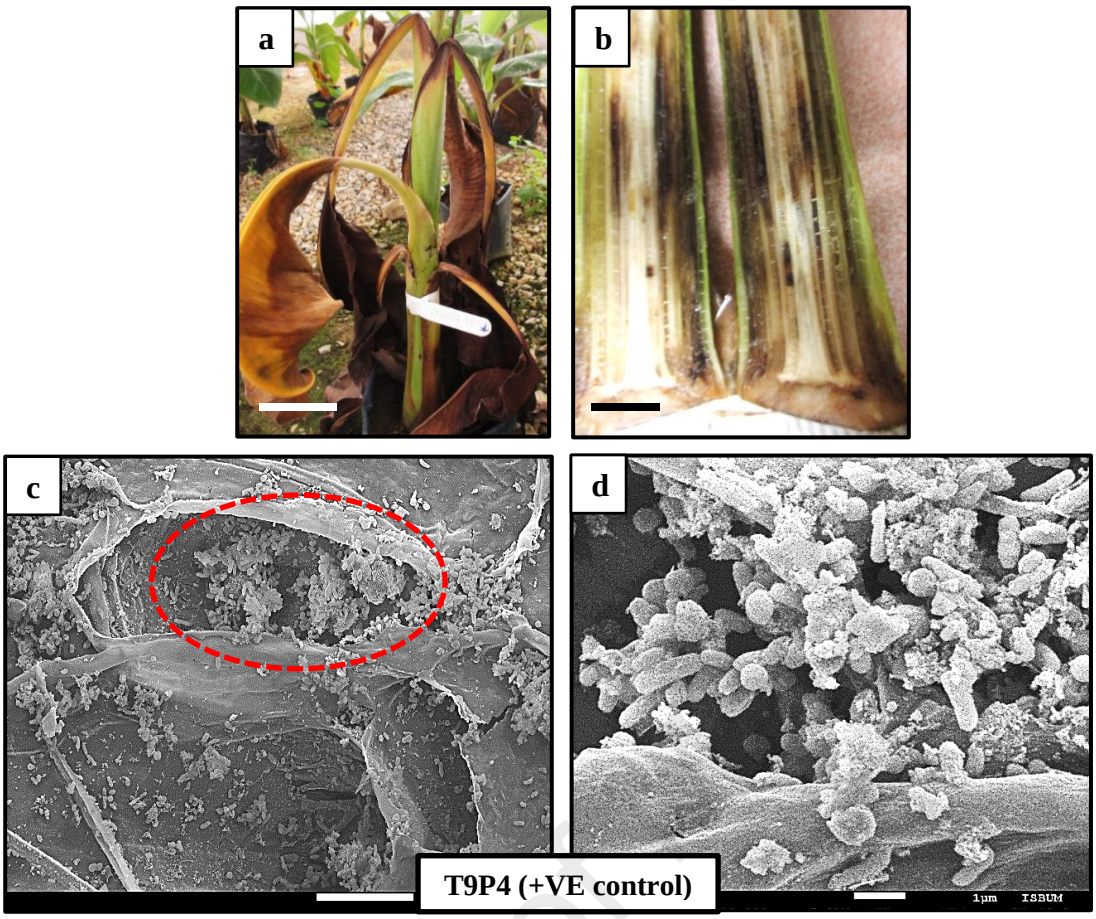

Figure 4.33: Representative of positive control plants (inoculated with pathogenic strain, BDB only) showing bacterial wilt symptoms, a) +ve control plant, T9P4 was severely wilt, b) cross-section of pseudostem part indicated discoloured and rotten tissues c) EPS-enmeshed bacterial clumps residing in the xylem vessels d) 10, 000X magnified plant tissues' surface revealed bacterial aggregates clumped in biofilm (scale bar, a =10 cm, b =2 cm, c =10 µM and d =1 µM).

Based on the SEM images of bacterial colonization and structural changes in plant tissues, treatment 5 which was inoculated with pathogenic strain, *R. syzygii* subsp. *celebesensis* and biofilm-former, *K. radicincitans* indicated the colonization of the most tightly-packed structures of EPS-encased bacteria forming a biofilm. Hence, *K. radicincitans* was chosen for subsequent study to further provide insights into the roles of this bacterium in association with bacterial wilt-diseased banana plant.

4.4 Whole genome sequence analyses of *Kosakonia radicincitans*, UMEnt01/12 a strain associated with bacterial wilt-diseased *Musa* spp.

4.4.1 Phenotypic characteristics of *Kosakonia radicincitans*

Kosakonia radicincitans UMEnt01/12 (SY-ST-17) strain was isolated from the stem tissues of the bacterial wilt-diseased banana plant with clear wilting symptoms where young leaves turned yellow and breakage of the petiole was evident. The vascular bundles in the pseudostems showed the appearance of narrow dark stripes. Based on the morphological characteristics of the bacterial colonies on TZC agar (Figure 4.34), the biofilm-forming ability (strong biofilm-former) of the strain and the evidence of bacterial colonization from *in-vivo* assessment in the glasshouse conditions, the SY-ST-17 strain was then selected for the whole genome sequence (WGS) analysis. The genome study was carried out to further identify the genetic features of the strain which are related to survivability, adhesion and colonization in the vascular tissues of banana plants, as well as the synergistic interaction and potential pathogenic properties of the bacterium that could enhance the severity of the host plant infection.

Figure 4.34: *Kosakonia radicincitans* UMEnt01/12 (SY-ST-17) strain on the Tetrazolium chloride (TZC) medium (scale bar, 2 cm).

4.4.2 Species identification

Comparison of 16S rRNA gene sequence of *K. radicincitans* UMEnt01/12 with 16S rRNA gene sequences of the bacterial strains with the top BLAST hits from the database showed that the UMEnt01/12 strain is closely related to a cluster containing *Kosakonia oryzae*, a nitrogen-fixing bacterium (Figure 4.35) with 99.0% nucleotide sequence similarity (E-value=0). However, among the members of *Enterobacteriaceae*, variation within the 16Sr RNA gene does not allow confident species identification (Mollet et al., 1997).

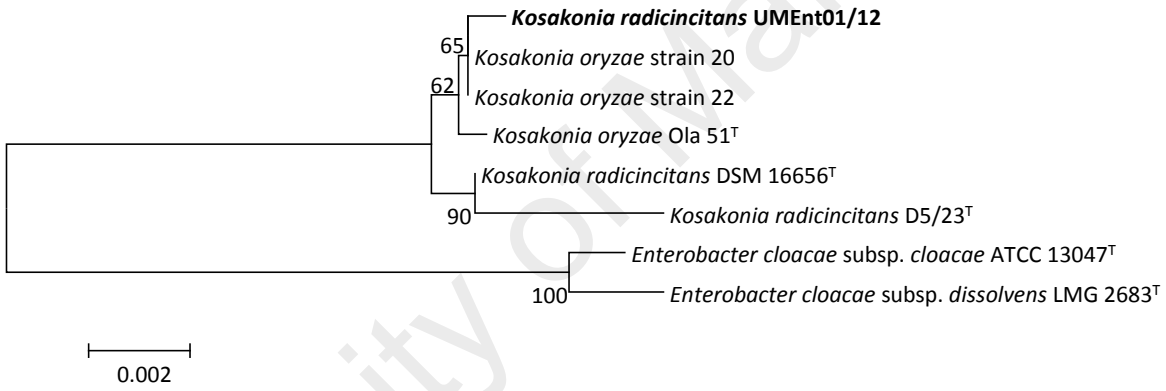

Figure 4.35: Phylogenetic analysis based on 16S rRNA gene sequences constructed after multiple alignments of eight nucleotide sequences with ClustalW Distances (distance options according to Kimura-2 model) and clustered with the Neighbor-Joining method (Saitou & Nei, 1987).

Hence, the RNA polymerase beta subunit encoding gene (*rpoB*) sequences have been used as an alternative marker for universal bacterial genotypic identification of the members of *Enterobacteriaceae* (Mollet et al., 1997). The *rpoB* sequence of UMEnt01/12 strain was aligned with *rpoB* sequence of the members of the family *Enterobacteriaceae* (Figure 4.36). Using the *rpoB* sequences, *K. radicincitans* UMEnt01/12 is closely

grouped with the other two *K. radicincitans* strains. Based on the NCBI BLAST results, the *rpoB* genes of *K. radicincitans* UMEnt01/12 showed 99.0% sequence similarity with *K. radicincitans* LMG 23767 (E-value=0).

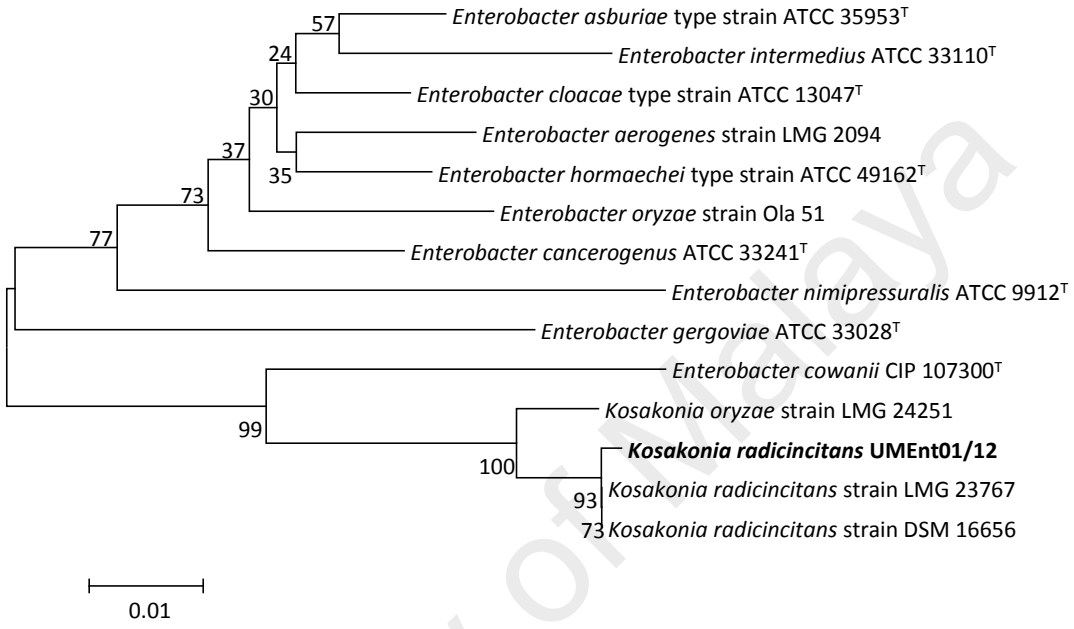

Figure 4.36: Phylogenetic analysis based on RNA polymerase beta subunit encoding gene (*rpoB*) gene sequences constructed after multiple alignments of 14 nucleotide sequences with ClustalW Distances (distance options according to Kimura-2 model) and clustered with the Neighbor-Joining method (Saitou & Nei, 1987).

4.4.3 Genome structure and general features

The genome size of the Gamma-proteobacterium, *K. radicincitans* UMEnt01/12 is 5, 783, 769 bp with a G+C content of 53.9% (Table 4.9). The analysis revealed a total of 5, 463 coding sequence (CDS), 75 tRNAs, and 9 rRNAs, which accounted for approximately 89.0% of the total genome size. Out of the 5, 463 CDS, a total of 4, 957 CDS (90.7% of the total CDS) could be assigned to putative biological functions, while another 283 CDS and 223 CDS are annotated as hypothetical proteins and unassigned functions,

respectively. For the CDS with unassigned functions, there are 13 CDS codes toxin/anti-toxin systems. For genes related to mobile elements, 13 and 21 CDS were found for prophage protein and integrases, respectively. CDS related to plasmid function (9 CDS) was also detected. Besides, operon systems for crucial maintenance of the cell's machineries are identified which include gene families of ABC (308), MFS (42), PTS (38) and RND (11). Interestingly, CDS related to pathogenicity, the Type IV and Type VI secretion systems were also found. On the contrary for the CDS with unassigned functions, 110 CDS (2%) had no homology to any previously reported sequence. These unassigned and uncharacterized CDS may possibly encode for unknown functions that confer fitness advantages to the organism and these would offer great opportunity for future function characterization. The subsystem category distribution based on the Rapid Annotation using Subsystem Technology (RAST) server and the SEED database is presented in Figure 4.37. The two most abundant features are carbohydrates and amino acids metabolism which represent 28.6% of total subsystem features counts.

Table 4.9: Summarized features of *Kosakonia radicincitans* UMEnt01/12 genome.

General traits	
Size (bp)	5 783 769
G+C content	53.90%
Assigned function (including putative)	4953
Toxin/anti-toxin systems	13
Phage shock protein	5
Prophage protein	13
Integrases	21
Plasmid function	9
ABC family	308
MFS family	42
PTS	38
RND	11
Type IV secretion protein RHS	11
Type VI secretion protein	33
Hypothetical proteins	283
No homology to any previously reported sequences	110

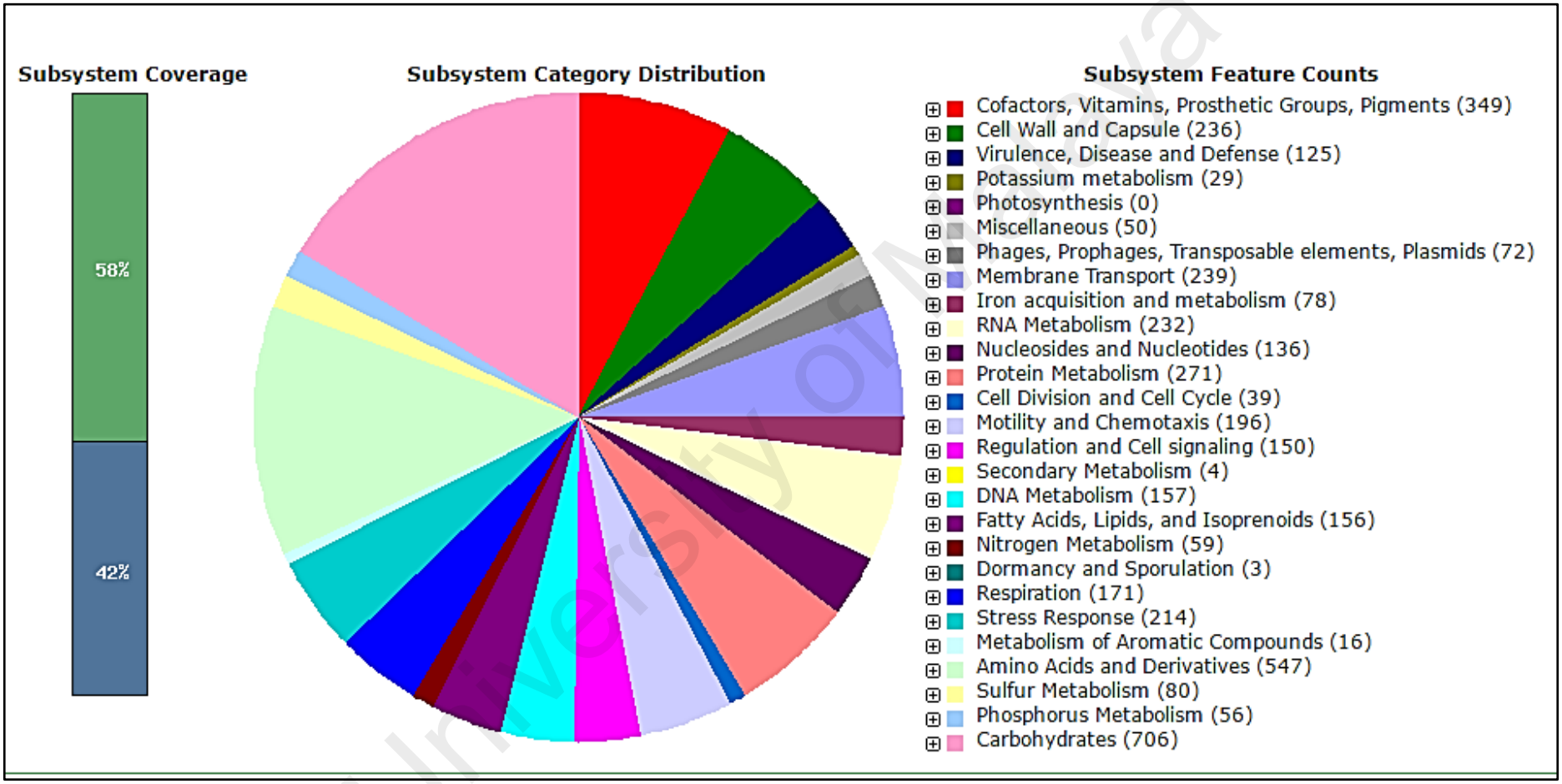

Figure 4.37 Subsystem category distribution and their feature counts for *Kosakonia radicincitans* UMEnt01/12 based on genome annotations performed using the RAST Annotation Server and the SEED database (Aziz et al., 2008; Overbeek et al., 2014).

The presence of genes related to toxin/anti-toxin (T/A) systems in the genome, which are usually transferable via plasmid or other genetic elements suggest that *K. radicincitans* UMEnt01/12 is potentially pathogenic. T/A system is commonly associated with pathogenic bacteria and often transferred through horizontal transfer and have been found harboured on plasmids conferring antibiotic-resistant and virulence genes. In addition, other toxin-antitoxin system such as the post-segregation antitoxin, *CcdA* and toxin *RelE*, and also TabA and HipA, which are involved in biofilm formation were identified in the genome, suggesting a complex interplay between toxin-antitoxin in the adaptation of the organism.

The genome of *K. radicincitans* UMEnt01/12 harbour putative clustered regularly interspaced short palindromic repeats (CRISPR) sequence, known as segment of prokaryotic DNA which contains short, repetitive base sequences. This CRISPR was identifies in Contig 21 (NZ_JDYJ01000021.1 and the sequence length is 125 bp with a spacer and a direct repeat consensus of 33 bp (Figure 4.38). Normally, small clusters of CRISPR-associated (cas) genes are located next to CRISPR sequences (Jansen et al., 2002). The CRISPR/Cas system is a prokaryotic immune system which is likely to be responsible for resistance properties against bacteriophages (Taghavi et al., 2010).

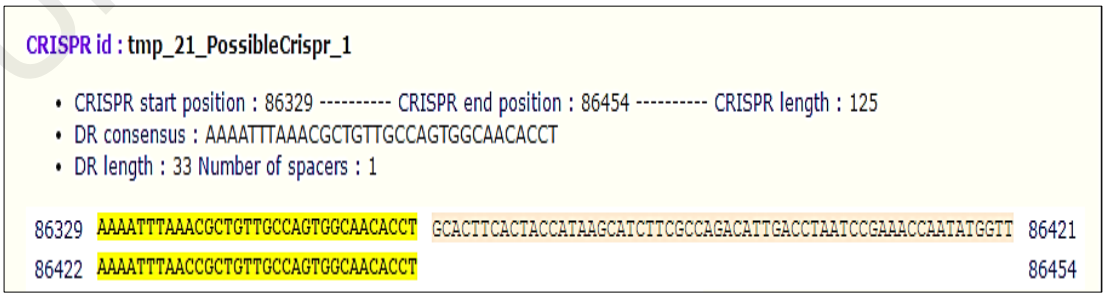

Figure 4.38: Possible CRISPR sequence detected in the genome of UMEnt01/12 strain using the CRISPR Finder (http://crispr.u-psud.fr/Server/CRISPRfinder.php) (Grissa et al., 2007).

4.4.4 Survivability and adaptation in the plant endosphere: *Kosakonia radicincitans* UMEnt01/12 metabolic capabilities

4.4.4.1 Carbohydrate metabolisms

This *K. radicincitans* UMEnt01/12 genome possesses proteins for the utilization of large varieties of plant derived compounds as carbon sources such as N-acetylglucosamine, L-arabinose, D-cellobiose, D-galactose, D-fructose, gluconate, D-glucose, D-maltose, D-mannose, D-xylose, D-sorbitol, D-mannitol, acetate, aconitate, citrate, fumarate, glutarate, DL-lactate, L-malate, pyruvate, L-aspartate, L-alanine, L-proline and L-serine, also found in *K. radicincitans* D5/23^T (Kampfer et al., 2005), with addition of proteins for assimilations of xylulokinase, inositol, putrescine, oxoglutarate, histidine, leucine, hydroxybenzoate and phenylalanine.

The genome of *K. radicincitans* also comprises of five subunits of malonate decarboxylases, known as alpha, beta, delta, gamma, and epsilon encoded by *mdcA*, *mdcD, mdcE, mdcC* and *mdcH*, respectively. The presence of a repertoire of sugar related genes may increase the versatility of the organism in utilising a broader range of sugar resources for better host adaptation. The presence of multiples copies of genes encoding glycoside hydrolases integrated in several contigs suggest the genes may have been duplicated for functional optimization.

4.4.4.2 Oxidative stress to counteract the plant's defence mechanism

The *K. radicincitans* UMEnt01/12 genome encodes three superoxide dismutases (SOD) known as, SodA, a manganese superoxide dismutase, the predominant enzyme during aerobic growth; SodB, an iron superoxide dismutase, which convert O_2 to H_2O_2 and O_2; and SodC, a Copper/Zinc superoxide dismutase, with properties like eukaryotic. Hence,

this bacterium might be able to synthesize enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione to protect itself against oxidative stress.

The genome of UMEnt01/12 also contains genes encoded for lipid hydroperoxide peroxidase, alkyl hydroperoxide reductases (subunit c and f), thiol-specific antioxidant, AhpC, organic hydroperoxides resistance protein, and glutathione s-transferase (GST) genes. GSTs are evolutionarily conserved enzymes that functions in the detoxification of most xenobiotic compounds (Veal et al., 2002) and protect the cells against reactive oxygen species (ROS) (Fouts et al., 2008).

Other glutathione-related proteins which also found in this genome are S-glutathione dehydrogenase class III alcohol dehydrogenase, five glutathione ABC transporters ATP-binding protein, hydroxyacylglutathione hydrolase, cysteine glutathione ABC transporter membrane ATP-binding component, glutathione-regulated potassium-efflux system protein KefBCF, glutathione transferase, glutathione peroxidase, glutathione reductase, glutathione synthase, ribosomal protein S-formylglutathione hydrolase, hydroxyacylglutathione hydrolase, and lactoylglutathionelyase. The presence of glutathione, (GSH) at high concentration help in maintaining a strong reducing environment in the cell, and its reduced form is maintained by glutathione reductase using NADPH as a source of reducing power while, glutathione peroxidase is functioning as a scavenging enzyme (Cabiscol et al., 2010).

This genome contains the class I plant peroxidase (intracellular peroxidase), cytochrome C peroxidase which probably protects against toxic peroxides, putative cytochrome D ubiquinol oxidases and pyruvate flavodoxin oxidoreductase, *nifJ* required for the transfer of electrons from pyruvate to flavodoxin, which reduces nitrogenase. The expression of the oxidative stress response system is coupled to other complex regulatory networks in

the cells (Storz & Imlayt, 1999). This *K. radicincitans* could be able to survive in an oxidative environment due to the presence of oxidative stress-resistance chaperone, *YajL*, oxidative stress defense protein, putative starvation proteins, phosphate starvation inducible protein, PsiF, stringent starvation protein, DNA starvation stationary phase protection and carbon starvation proteins.

4.4.4.3 Resistance to heavy metal

The genome of *K. radicincitans* UMEnt01/12 carries genes which encode for proteins putatively involved in copper resistance and homeostasis, such as P-type ATPase CopA, the multicopper oxidase CueO, operon coding for the CopC and CopD copper resistance proteins, copper homeostasis protein, CutC and lipoprotein involved with copper homeostasis and adhesion, NlpE. The genome also encodes for arsenical pump and resistance operon repressor, arsenate reductases, tellurite resistance proteins TehAB, and chromate transporter.

Heavy metals are also important cofactors, and this bacterial genome encodes several genes involved in heavy metal transport and efflux. Genes were found for transport and efflux of zinc, magnesium, nickel and cobalt. Other heavy metal resistance genes located in the genome were encoded for heavy metal-translocating P-type ATPase which involved in Cd/Co/Hg/Pb/Zn resistance, heavy metal RND transporter, and heavy metal sensor kinase family protein. *K. radicincitans* genome also comprises of proteins coded for cobalt-precorrin, *cobGJLM* and *cbiCETDJ* and cobalt transporters. Although this bacterium was isolated from the endosphere of banana plant, the presence of these genes may provide an additional advantage to survive in the other environment such as soil, where these metals might be present.

4.4.4.4 Uptake of plant nutrients

UMEnt01/12 possesses 638 ORFs which encode for putative transporter genes and the distribution of transporter families are almost similar to the members of the *Enterobacteriaceae*. For example, the genome encodes 308 ATP-binding cassette (ABC) family transporter genes, 42 major facilitator superfamily (MFS) genes, 38 phosphotransferase system (PTS) family genes and 11 resistance nodulation and cell division (RND) family transporter genes. Transporters in ABC and MFS families are commonly involved in the uptake of various nutrients, such as sugars, amino acids, peptides, nucleosides and various ions, as well as the extrusion of metabolite waste, toxic by-products and antibiotics (Fouts et al., 2008). Meanwhile, the PTS transporters in UMEnt01/12 genome might be functioning in the uptake of α-glucoside, β-glucoside, glucose, maltose, fructose, cellobiose, trehalose, acetylmuramic acid, mannose and other few more substrates (Taghavi et al., 2010).

4.4.5 Bacterial-host and endophyte-pathogen interactions

4.4.5.1 Biofilm formation in the host plant

Biofilm formation proceeds through a series of programmed steps. The biofilm development initially required flagellar motility and type IV pili-mediated twitching for surface attachment and microcolony aggregation (O'Toole & Kolter, 1998). As the bacterial cells formed the microcolonies, a mechanism of cell-cell signalling known as quorum sensing has been postulated to play an important role in the development of matured biofilm (Davies et al., 1998; Walker et al., 2004). Some putative biofilm formation-related proteins found in this UMEnt01/12 bacterial genome are biofilm formation regulatory protein, BssR and also biofilm stress and motility protein A.

4.4.5.2 Motility and chemotaxis

Motility is an important features for the endophytic bacteria which assist in the movement of the bacteria inside the host plant to colonize specific plant parts (Taghavi et al., 2010) and also suggested to be involved in the biofilm formation (O'Toole & Kolter, 1998). *K. radicincitans* UMEnt01/12 is well-equipped with flagellar biosynthesis operons (*flgABCDEFGHIJKLNM*, flagellar transcriptional regulator, *flhCD*, flagellar motor, *motAB*) and a group of flagellin genes (*fliACDEFGHJKLMOPQR*).

4.4.5.3 Lipopolysaccharides biosynthesis

The structure of lipopolysaccharides (LPS) is comprised of the Lipid A (or endotoxin), the core oligosaccharide (core OS) and the distal polysaccharide (O-antigens) (Nakao et al., 2012; Raetz & Whitfield, 2002). In *K. radicincitans* UMEnt01/12, the presence of these three components were detected. Some genes involved in LPS biosyntheses were also identified such as LPSABC transporter complex (*lptBFG*), involved in the translocation of LPS from the inner membrane to the outer membrane, and LPS biosynthesis genes, *rffA*. Three L-glycero-D-manno-heptose, (heptosyltransferase), called HepI, HepII, and HepIII were also found in the genome of *K. radicincitans* UMEnt01/12.

4.4.5.4 Cell-to-cell signalling: Quorum sensing

Biofilm development and the resulting intimate interactions with plants often require cell-cell communication between colonizing bacteria (Danhorn & Fuqua, 2007). The acylated homoserine lactones (AHLs) is the most common signal used for cell-to-cell communication in Gram-negative bacteria (Fuqua et al., 2001; Nasser & Reverchon,

2007). The basic molecular system of AHL-quorum sensing is mediated by the enzyme responsible for the AHL synthesis (LuxI-type proteins) and a transcriptional regulator (LuxR-type proteins), the activity of which is modulated by AHL (Fuqua et al., 2001). The AHL synthase families were not detected in *K. radicincitans* UMEnt01/12 strain. However, seven bacterial transcriptional regulators, LuxR family proteins, which are responsive towards the synthesized AHL were found in this UMEnt01/12 genome. The function of LuxR homologues as quorum sensors is mediated by the binding of N-acyl-L-homoserine lactone (AHL) signal molecules to the N-terminal receptor site of the proteins (Koch et al., 2005).

4.4.5.5 Synergistic interactions with the host plant

Most biological nitrogen fixation processes are controlled by the activity of molybdenum nitrogenase, found in all diazotrophic microorganisms (Rubio & Ludden, 2008). Nitrogen fixation genes (*Nif*) are the responsible gene for the coding of proteins associated with the fixation of atmospheric nitrogen into a form of nitrogen available to plants (Saikia & Jain, 2007).

The presence of *nif* operon, comprising the *nifYUBAMSNEHXWZQ* genes, in UMEnt01/12 strain suggested that this strain has the potential to biologically fix atmospheric nitrogen. The *nifENUSWXBYQ* genes encode proteins involved in the assembly and incorporation of the Fe and Mo into the nitrogenase subunits. These nitrogenase molybdenum-iron (MoFe) protein alpha- and beta-chain, are also found in *E. cloacae. nifA* is a regulatory protein in-charge of regulating the *nif* genes expression. *NifH* is one of nitrogenase structural gene and *nifM* appears to be an accessory protein for *nifH. nifZ* is required for the maturation of the nitrogenase MoFe protein. Histidine ammonia-lyases (HAL) was also detected in the chromosome of *K. radicincitans* UMEnt01/12.

Other ammonia lyses found were ethanolamine, L-serine and aspartate, which belong to the family of carbon-nitrogen lyases (Asano et al., 2004).

4.4.6 Potential pathogenic characteristics of *Kosakonia radicincitans* UMEnt01/12

4.4.6.1 Adhesion and colonization of the host plant endosphere

In *K. radicincitans* UMEnt01/12 strain, several genes which encode putative adhesion proteins were identified. For instance, type V secretory adhesion, also known as AIDA (adhesin involved in diffuse adherence) which could be assisting in biofilm formation (Hayashi et al., 2001), outer membrane adhesin-like protein, six hemagglutinin-related proteins, Type IV pilin biogenesis protein and six fimbrial-like adhesion proteins are some of the protein encoding genes found in the genome. *K. radicincitans* UMEnt01/12 also has a number of pili assembly chaperone PapD, pili assembly N- and C-terminal domain protein.

The genome of UMEnt01/12 contains genes encoding for degradation of pectate which could play a role in colonizing the interspatial region between plant cells. Pectin acetylesterase, pectate disaccharide-lyase (PATE), and pectatelyase, PelB which are involved in eliminative cleavage of pectate, yielding oligosaccharides with 4-deoxy-alpha-D-mann-4-enuronosyl groups at their non-reducing ends were also found. Operons responsible for bacterial cellulose biosynthesis were also identified such as the genes encodes for cellulose synthase catalytic subunits, cellulose synthase proteins and cellulose biosynthesis proteins. The bacterial cellulose production may possibly enhance the adhesion of this bacteria to the plant tissue (Taghavi et al., 2010).

4.4.6.2 Candidate genes responsible for pathogenesis

The genome of *K. radicincitans* UMEnt01/12 was found to code for several proteins putatively involved in pathogenesis. Type II secretion system (T2SS) formerly known as general secretory pathway was found in this bacterial genome together with approximately eleven Type IV secretion proteins, Rhs. This UMEnt01/12 strain also contains putative virulence factor (MviM), virulence associated protein (VagC), nitrogen fixation virulence-island protein (ImpE), myosin light chain kinase that assist in adherence of bacteria to the host, virulence factors, *srfABC* where the exact function of the *srfABC* operon remains unclear, but it is believed to be involved in host colonization (Worley et al., 2000).

4.4.6.3 Type VI secretion system (T6SS)

K. radicincitans UMEnt01/12 carries multiple T6SS clusters in its genome including the Hcp1 and Vgr family proteins. Other proteins found which related to the T6SS include EvpB family type VI secretion protein, DotU protein which is a component in Dot-Icm complex, and ImpA family type VI secretion-associated protein. To date, no study has shown the presence of a well-characterized T6SS in the genus of *Kosakonia*. Therefore, the function and expression of this system in *K. radicincitans* UMEnt01/12 have yet to be explored.

4.4.7 Comparison between genomic contents of closely related, *K. radicincitans* UMEnt01/12 and DSM 16656

A comparison of the *K. radicincitans* UMEnt01/12 genome with the closest genome (symmetrical identity: 90.62%) of entirely sequenced *K. radicincitans* DSM 16656 (NZ_AKYD00000000.1) (Witzel et al., 2012) revealed additional unique genes involved in adhesion, stress response, phage proteins, as well as many other regulators. The percentage of additional genes found in this genome which absence in DSM 16656 strain is approximately 5.3%. In particular, these additional genes of *K. radicincitans* UMEnt01/12 share the highest homology with that of the opportunistic plant pathogen, *E. cloacae* (~20%), followed by *Enterobacter* sp. 638, and *Escherichia coli*. There are about ~135 of hypothetical proteins with high similarity to *E. coli*, *E. cloacae,* and *S. enterica*.

Homologous proteins with *E. cloacae* are mostly identified as hypothetical proteins, membrane-related proteins, transporters, regulatory proteins, phages, stress-related proteins and metabolism-related proteins such as thiamine biosynthesis, molybdopterin biosynthesis, phosphomethylpyrimidine synthase and purine biosynthesis. There are also CDS identified as conjugative/ conjugal transfer proteins, TrbABCI and TraIDTWVKEL mostly found in *E. cloacae* subsp. *cloacae* and *Enterobacter* sp. R4-368.These proteins are secreted by the type IV secretion system (T4SS).

Both *K. radicincitans* UMEnt01/12 and *E. cloacae* shared genes which encode for Gnt-II system L-idonate transporter, IdnT, GntR family transcriptional regulator, phosphoribosylamine-glycine ligase, UDP-N-acetylenolpyruvoylglucosamine reductase, cytochrome C biogenesis family protein, DsbD and levansucrase regulator.

4.5 Diversity of bacterial communities associated with symptomatic and non-symptomatic bacterial wilt-diseased banana plants

4.5.1 Sampled plants

The symptomatic banana plants showed symptoms of yellowing and wilting of leaves, forming a skirt of dead leaves around the pseudostem. In contrast, the non-symptomatic plant appeared healthy and unaffected by the BW infection. The vascular bundles in the pseudostems of the symptomatic plants (SA and SB) were discoloured and blackened while, the pseudostem tissues of the non-symptomatic plant (NS) was free from any disease symptoms (Figure 4.39).

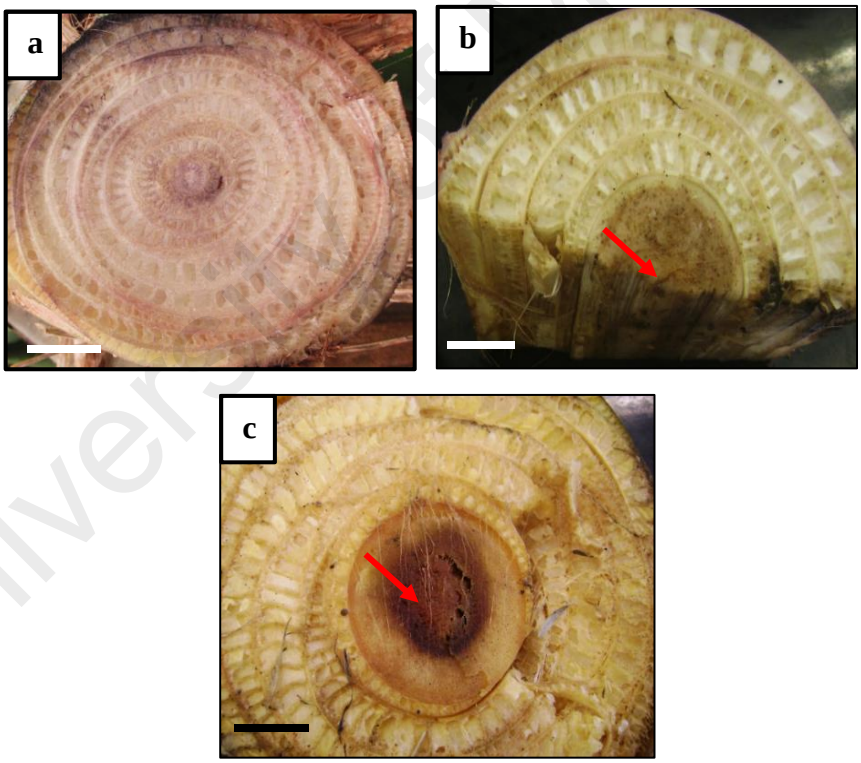

Figure 4.39: Cross-section of pseudostem tissues of (a) non-symptomatic (NS) (b) Symptomatic A (SA) and (c) Symptomatic B (SB) banana plants. The vascular bundles of pseudostem tissues of SA and SB plants were discoloured and blackened while the pseudostem tissues of NS plant did not show any discolouration. Red arrows indicate the symptoms (scale bar, 5 cm).

Pseudostem tissues of NS, SA and SB plants tested directly with the immunoassay strips specific for *R. solanacearum* gave positive results for the presence of the pathogenic bacteria, as indicated by the appearance of control line and test line on the strips (as shown in Figure 4.3).

4.5.2 Total genomic DNA extraction by conventional method

The DNA isolation method by Rahman et al. (2010) produced highly concentrated total genomic DNA (approximately 70 – 90 ng/µl) but of low quality, with average A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.5 – 2.0 and < 0.5, respectively. Although, the A_{260}/A_{280} ratios obtained from the plant samples extracted with this protocol were within 1.5 – 2.0, the A_{260}/A_{230} ratios were still far below 2.0.

In contrast, the DNA extraction protocol modified from Fan and Gulley (2001) and Souza et al. (2012) yielded higher quality and quantity of total genomic DNA which is of sufficient concentration and purity for 16S rRNA metagenomics analysis. Based on Nanodrop™ spectrophotometer reading, the average of 1.8 – 2.0 and > 1.8, were recorded for A_{260}/A_{280} and A_{260}/A_{230} ratios, respectively. The reading also indicated average concentration of 20 – 50 ng/µl for all the samples of extracted DNA.

4.5.3 Diversity indices and rarefaction curves

A total of 32.25 million raw reads were produced from a single next generation sequencing run with GC content of 53 – 54%. With subsequent refinement including gap closure and sequence quality improvement (finishing), this number of raw reads was reduced to 27.36 million reads for downstream analysis. From the BLAST results,

approximately 14.99 million sequences were categorized as root of MEGAN which were further classified into phyla and down to possible species level.

Rarefaction curves for each sample were constructed based on percent identity of matches more than 97% to indicate the sampling completeness. The rarefaction curves for SA and SB samples were approaching completeness, as depicted by the graphic curve plateaus (Figure 4.40). Any further sequencing of these two samples would produce very few new numbers of reads. However, the rarefaction curves for NS sample showed that further sequencing was necessary to be confident that the bacterial profile was completed (Figure 4.40). Based on the Shannon-Wiener and Simpson's diversity indices, the microbial population of non-symptomatic (NS) banana plant was most diverse with values of 3.160 and 4.107, respectively, compared to the other two symptomatic plants (SA: 2.177 and 2.599; SB: 0.028 and 1.004). NS was also the richest with 167 bacterial genera compared with SA (79 bacterial genera), and SB (44 bacterial genera) (Table 4.10).

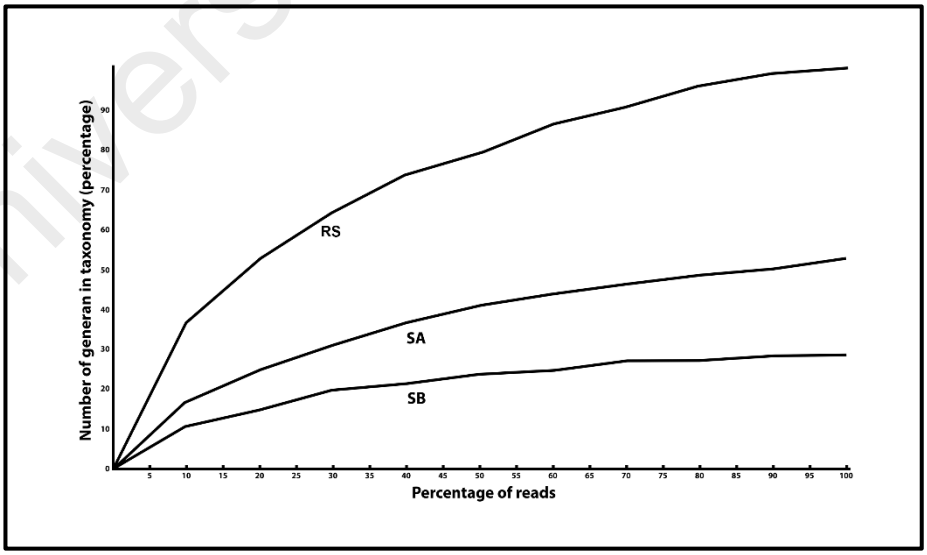

Figure 4.40: Rarefaction curves for the three samples, NS, SA, and SB showing the percentage of number of genera as a function of the percentage of reads, indicating sampling completeness. NS = Non-Symptomatic plant tissues, SA and SB = Symptomatic plants tissues

Table 4.10: Diversity indices of the non-symptomatic (NS) and symptomatic (SA and SB) plant tissues samples

Diversity index*	Sample		
	NS	SA	SB
Shannon-Wiener	3.160	2.177	0.028
Species Richness	167	79	44
Simpson's Diversity	4.807	2.599	1.004

*Diversity indices (Shannon-Wiener and Simpson's diversity) and species richness at the genera level were calculated using the MEGAN4 software.

4.5.4 Taxonomic diversity

All the post-filtered reads for each sample (NS, SA, and SB) were identified using the NCBI 16S microbial database according to the confidence threshold in Metagenome Analyzer (MEGAN4) for each taxonomic level as described in Table 4.11. Based on the taxonomic classification, all of the 16S rRNA gene fragments for the three samples were assigned to Bacteria. These fragments were further classified into a total of seventeen and nine known bacterial phyla for the non-symptomatic (NS) and symptomatic (SA and SB) plants, respectively.

Table 4.11: Taxonomic designations based on confidence threshold cut-off, a bootstrap-like confidence estimate.

Taxonomic Designation*	Confidence threshold cut-off (%)
Phylum	>80
Class	>85
Order	>90
Family	>95
Genus	>97
Species	>99

*Post-filtered reads were classified from phylum to genus using the NCBI 16S microbial database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Cyanobacteria made up the largest division in NS and SA samples (97% and 98%, respectively). It is important to note that, some Cyanobacteria could not be identified down to the order level, therefore the number of reads assigned to each classified orders were lower as compared to the number of reads in the phylum level. Nevertheless, in both NS and SA samples, this phylum comprised mainly of Chroococcales (58.6% and 56.2%, of the total Cyanobacteria, respectively) and Nostocales (36.5% and 38.4%, respectively), with Oscillatoriales and Pleurocapsales being present in smaller proportions (both accounted for 4.9% and 5.4%, respectively).

The second most abundant phylum in NS and SA samples was Proteobacteria which accounted for 2.1% and 1.4% of the total 16S rRNA gene fragments, respectively. Among the classes of Proteobacteria, Alphaproteobacteria was the most abundant found in both plant samples (66.9% and 49.5% of the total Proteobacteria in NS and SA samples, respectively), followed by Betaproteobacteria (27.7% and 48.2%, respectively), Gammaproteobacteria (4.4% and 2.1%, respectively) and Deltaproteobacteria (1% and 0.2%, respectively). Another class, Epsilonproteobacteria (0.03%) was only present in NS sample and these fragments were unable to be assigned to the lower taxa.

On the other hand, Proteobacteria (81.5%) was found as the largest phylum of Bacteria present in SB sample. This phylum was dominated by β-proteobacteria, which accounted for more than 99.8% of the total Proteobacteria in the SB tissue sample. Meanwhile, α-, γ- and δ-Proteobacteria were existed in minute proportions (<0.2%). Cyanobacteria (18.4%) was detected as the second most common phylum in this symptomatic (SB) plant sample (Table 4.12). The distribution of orders in this phylum include Chroococcales (40.6% of the total Cyanobacteria), followed by Nostocales (32.8%), Pleurocapsales (23.2%), and Oscillatoriales (3.4%). The remaining phyla in all these plant samples (NS, SA and SB) represented less than 0.5% of the total population. However, NS plant sample was observed to have the most diverse phyla (Figure 4.41).

Phyla that were present only in NS were Fusobacteria, Verrucomicrobia, Chloroflexi, Fibrobacteres, Gemmatimonadetes, Nitrospirae, Tenericutes and Spirochaetes.

Table 4.12: Percentage of bacterial phyla associated with NS, SA, and SB tissue samples.

Phyla*	**Sample Designation****		
	NS	**SA**	**SB**
Actinobacteria	0.12	0.05	0.007
Aquificae	<0.002	<0.002	ND
Bacteroidetes	0.19	0.09	0.08
Verrucomicrobia	<0.002	ND	ND
Chloroflexi	<0.002	ND	ND
Cyanobacteria	97.49	98.44	18.41
Deinococcus-Thermus	<0.002	<0.002	0.002
Acidobacteria	0.05232	0.01497	<0.002
Fibrobacteres	<0.002	ND	ND
Firmicutes	0.012	0.006	0.002
Fusobacteria	<0.002	ND	ND
Gemmatimonadetes	<0.002	ND	ND
Nitrospirae	<0.002	ND	ND
Planctomycetes	0.004	<0.002	ND
Proteobacteria	2.13	1.39	81.49
Spirochaetes	<0.002	ND	ND
Tenericutes	<0.002	ND	ND

*Phylum taxonomy and abundance were classified based on confidence threshold cut-off of >80% using the NCBI 16S microbial database. Values are given as percentages.

**NS = non-symptomatic plant tissues, SA and SB = symptomatic plant tissues ND = Not detected.

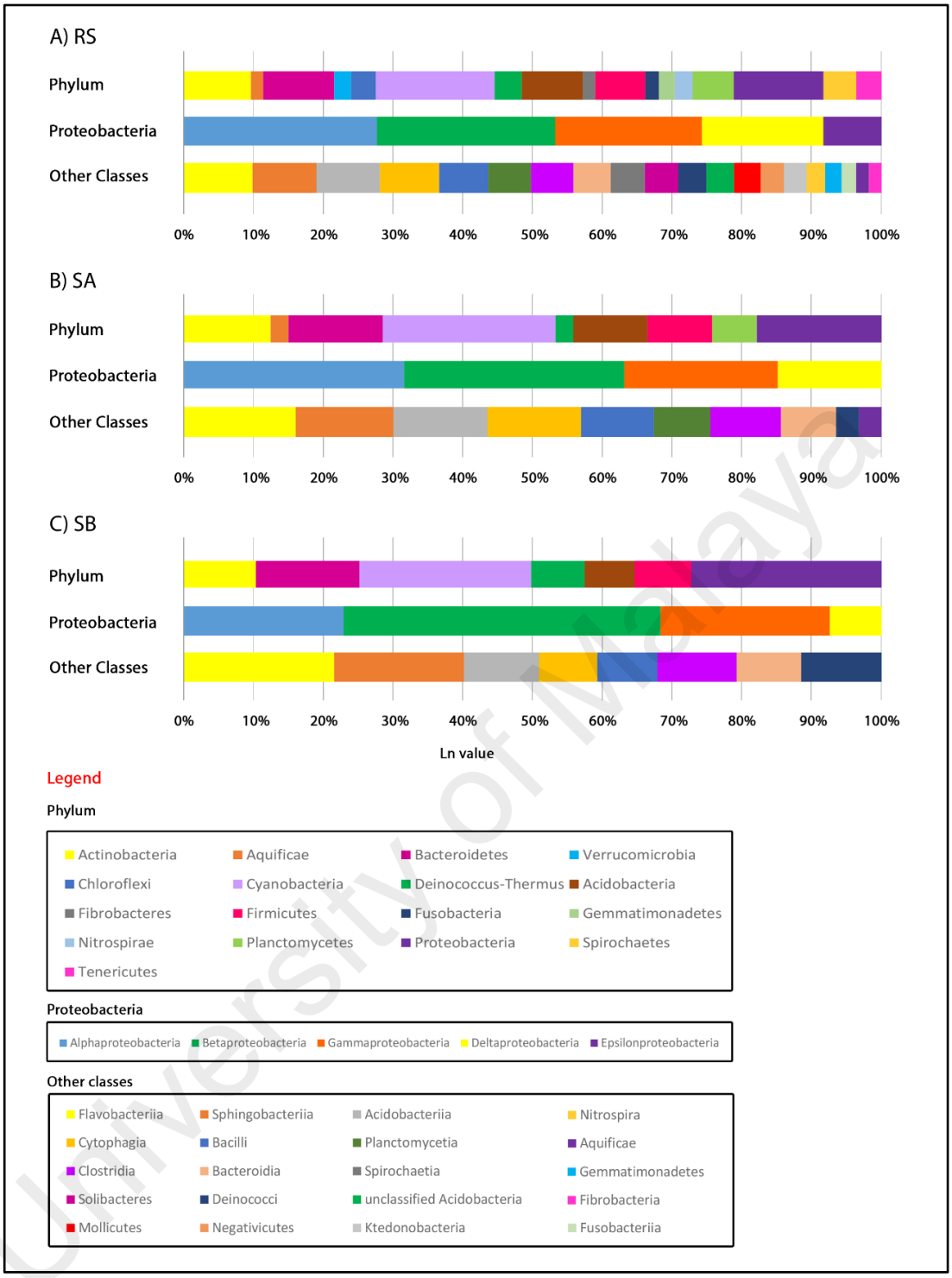

Figure 4.41: Taxonomic diversity and relative abundance at the phylum and class level of bacteria associated with NS, SA and SB tissue samples. Phyla were identified on the basis of confidence threshold cut-off of > 80%, and classes on a confidence threshold cut-off of > 85%, using the NCBI 16S microbial database. Phylum and class distributions are mentioned as natural log (ln) value of each number of reads. NS = Non-Symptomatic plant tissues, SA and SB = Symptomatic plants tissues.

Based on the taxonomic diversity classifications (Figure 4.41), all classes of Proteobacteria, Flavobacteriia, Sphingobacteriia, Deinococci, Clostridia, Acidobacteriia, Bacteroidia, Bacilli, and Cytophagia were present in all the three samples at varying abundance. Interestingly, the two symptomatic plant tissues, SA and SB were having more similar number and type of bacterial classes as compared to those found in the non-symptomatic plant tissues, NS.

At the Order level, members of Rhizobiales accounted for the highest number of fragments in non-symptomatic (NS) plant sample (35.3% out of the entire order) with few families including Bradyrhizobiaceae, Hyphomicrobiaceae and Rhizobiaceae which comprised of bacteria related to nitrogen-fixing, legume-nodulating and microsymbiotic relationships (Black et al., 2012; Ramírez-Bahena et al., 2013). In the symptomatic (SA and SB) plant samples, Burkholderiales was the most dominant order (41.2% and 99.7%, respectively) with the leading families of Burkholderiaceae (accounting for 99.7% of Burkholderiales fragments). Taxa placed into Burkholderiaceae included generally aerobic, rod-shaped, Gram-negative phytopathogens, opportunistic pathogens and primary pathogens for humans and animals (Coenye, 2014).

Our data showed that the three leading families found in NS and SA plant samples were Burkholderiaceae, Methylobacteriaceae and Sphingomonadaceae which accounted for more than 75% of the entire families of these two samples. In SB plant, Burkholderiaceae dominated the sample with more than 99% of the total 16S rRNA gene fragments assigned to this family. At the Genus level, *Ralstonia*, *Sphingomonas*, *Methylobacterium*, *Flavobacterium*, and *Pseudomonas* represented the five major genera found in those three tissue samples (which accounted for 84.3%, 91.6% and 99.9% of the entire set of genera in NS, SA and SB, respectively).

4.5.5 Comparisons of bacterial genera in non-symptomatic (NS) and symptomatic (SA) plant samples from the same geographical location

A Venn diagram (Figure 4.42) was further constructed by considering bacterial populations at the genus level with confidence threshold cut-off of > 97%. Based on the Venn diagram of NS and SA, 69 genera were assigned to both non-symptomatic and symptomatic plants samples (Figure 4.42). In non-symptomatic (NS) plant, majority of the gene fragments were assigned to *Ralstonia* (33.5% of the total genera), followed by *Sphingomonas* (24%) and *Methylobacterium* (17.9%). Whereas in SA plant, *Ralstonia* still accounted for the highest percentage of the total genera (approximately 58%), followed by *Methylobacterium* (19.8%) and only 7.2% of *Sphingomonas* was detected in this plant sample. Based on these data, it is clearly showed that *Ralstonia* was more abundant in SA than in NS plant sample. Another dominant genus, *Sphingomonas* was far more abundant in the NS as compared to the SA plant sample. The *Methylobacterium* represented approximately equivalent proportions in both plant samples.

The Venn diagram (Figure 4.42) also reveals that 102 bacterial genera were only assigned to the non-symptomatic plant (NS). Diverse genera of Actinobacteria, Bacteroidetes, Acidobacteria, Firmicutes and Proteobacteria were represented in this particular group. In total, these genera accounted for approximately 4% of the total 16S rRNA gene fragments of the NS plant sample. On the other hand, the endophytic communities associated with symptomatic plant were noticeably lower in diversity and abundance compared with those in the non-symptomatic plant.

4.5.6 Comparisons of bacterial genera in two symptomatic plant samples (SA and SB) from different geographical location

Another Venn diagram (Figure 4.43) was constructed to compare between the two symptomatic banana plants from different geographical areas, SA and SB. Based on the Venn diagram of SA and SB, 35 genera were assigned to both symptomatic plants. Most of these genera belong to the phylum of Proteobacteria. Figure 4.43 also indicates that the SA plant was inhabited by more diverse bacterial populations as compared to the SB plant. In the SB plant sample, *Ralstonia* was the most abundant genus with more than 3 million 16S rRNA gene fragments assigned to this genus (equals to more than 99% of total bacterial genera). Other bacterial genera accounted for less than 0.5% of the 16S rRNA gene fragments assigned to this SB plant.

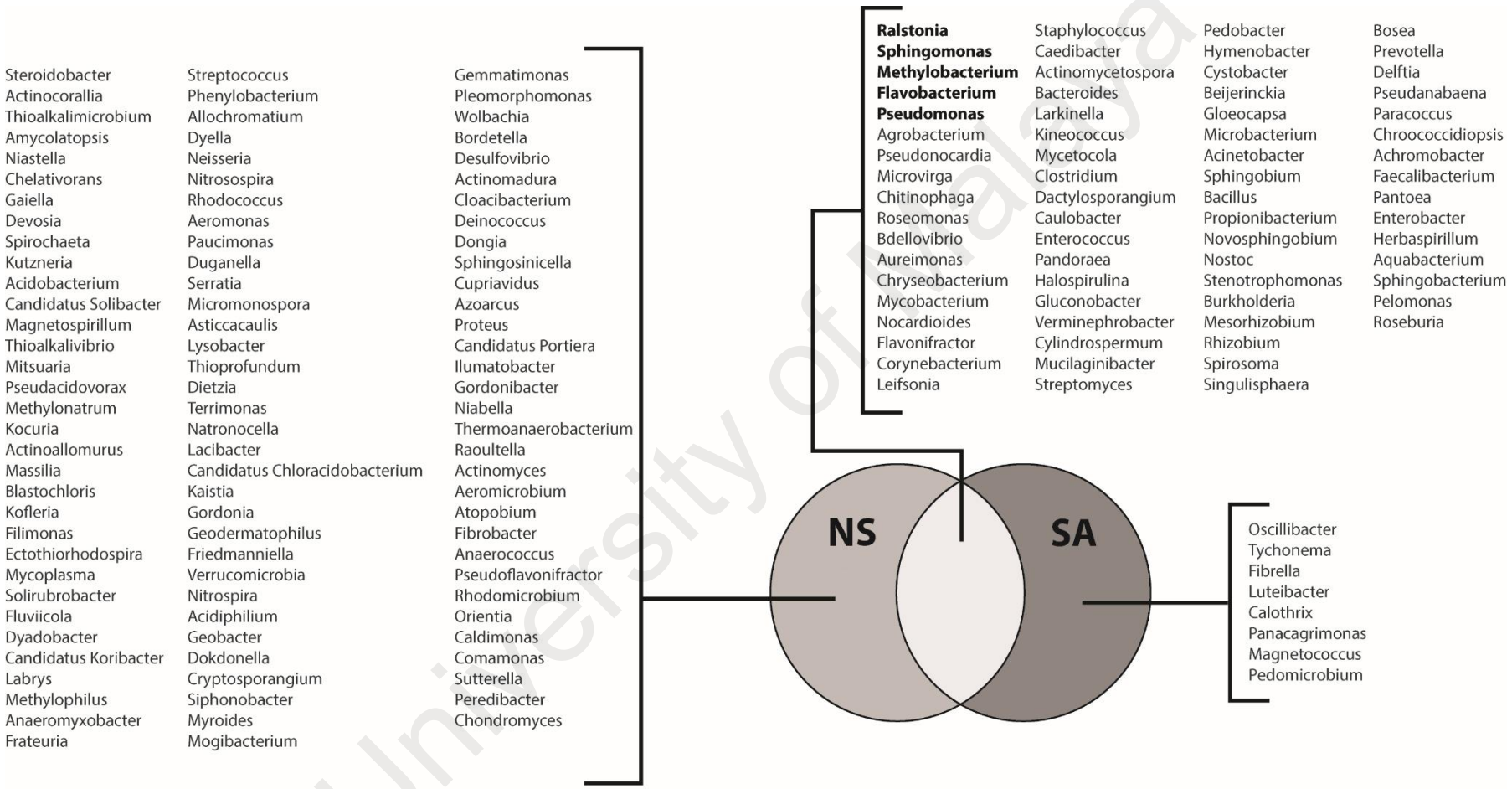

Figure 4.42: Venn diagram comparison of genera of bacterial population unique to each non- symptomatic (NS) and symptomatic (SA) plant tissues samples and shared by both (NS and SA) tissue samples. Genera were classified on the basis of confidence threshold cut-off of >97%.

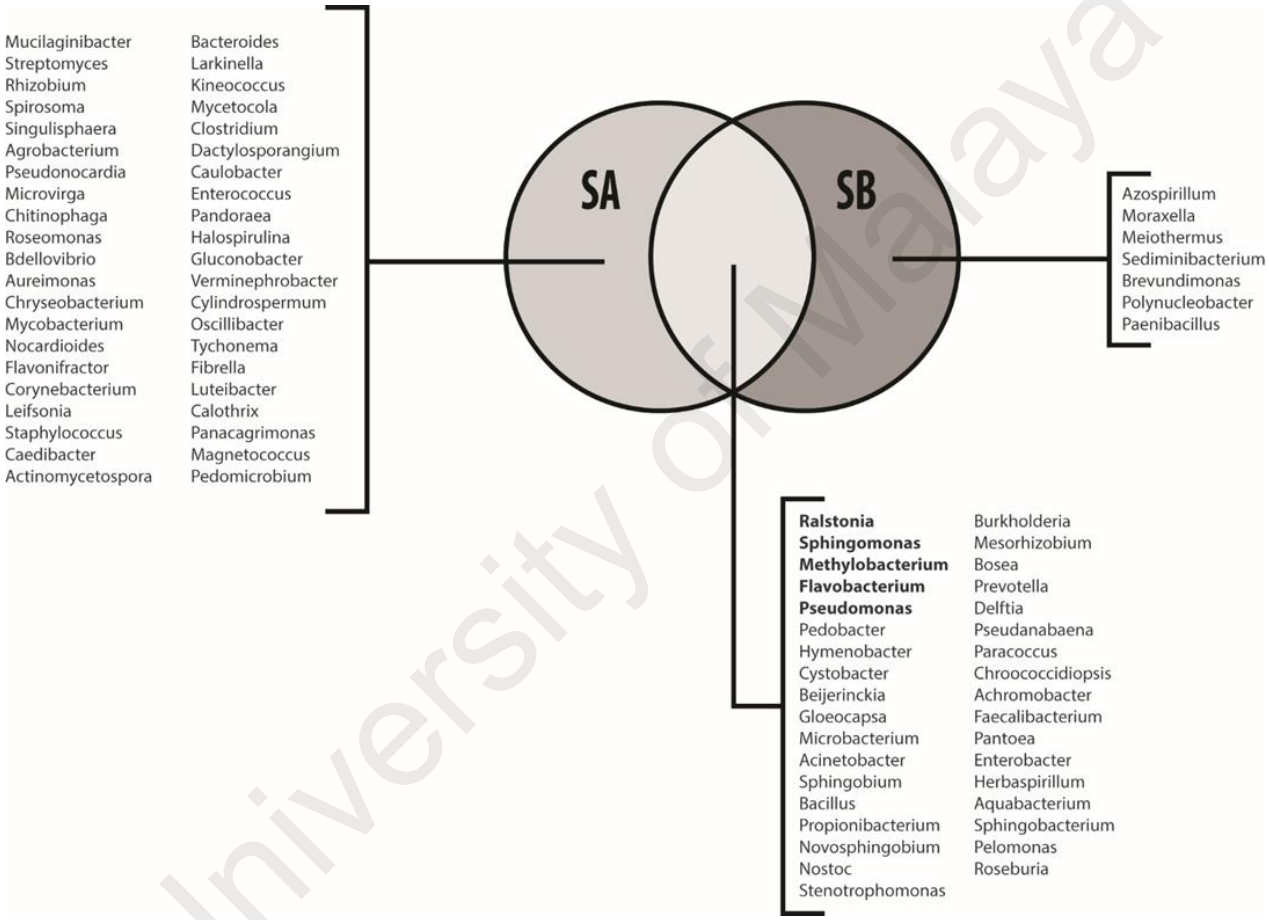

Figure 4.43: Venn diagram comparison of genera of bacterial populations unique to and shared by each symptomatic A (SA) and symptomatic B (SB) plant tissues samples. Genera were classified on the basis of confidence threshold cut-off of >97%.

The network comparison based on ecological indices (Simpson's and Shannon-Wiener diversity and species richness) was performed to anticipate the non-rooted relationship between these three plant samples using taxonomic results and number of 16S rRNA gene fragments assigned in the analysis. As a result, microbial population in the symptomatic plants, SA was closely related to the SB which was distinctly different from that of the non-symptomatic plant, NS. This comparison indicated that the diversity of bacterial communities in the two symptomatic plants (SA and SB) were almost similar regardless of the geographical location of the samples collected. The non-symptomatic (NS) plant showed some divergence from the symptomatic one (SA) despite the fact that the two plants were collected from the same sampling area.

CHAPTER 5: DISCUSSION

5.1 Identification and characterization of the isolated bacteria from bacterial wilt-diseased banana plants using the conventional method

A quick diagnostic test to identify the plant with bacterial wilt (BW) disease was necessary because plant wilting caused by bacteria can be confused with symptoms induced by other pathogens, insects or mechanical damage at the stem base or due to drought (Champoiseau et al., 2010; Muthoni et al., 2012). Therefore, infection by bacteria was confirmed by placing the cut pseudostem sections in a beaker of water. Viscous smoke-like threads of bacterial ooze were observed streaming from the symptomatic vascular bundles of the banana plants (Figure 4.2d). This streaming test was a valuable diagnostic tool for rapid confirmation of BW disease in field (Champoiseau et al., 2009).

The ImmunoStrip for Rs showed positive result for presence of *Ralstonia solanacearum*. This serological test uses Rs-specific monoclonal antibody (MAb) that reacts with an extracellular polymeric substances or exopolysaccharides (EPS) antigen (Paret et al., 2010). The limit of detection of the kit is around 10^5 cfu/ml of bacteria. Appearance of test line and control line indicate presence of *R. solanacearum* species complex strains according to the manufacturer's recommendation (shown in Figure 4.3). The PCR amplification using the non-specific, Y2 and specific, OLI-1 primers facilitated in detection of not only *R. solanacearum* species complex but also the other two closely related species, *Ralstonia syzygii* subsp. *celebesensis* (formerly known as Blood disease bacterium, BDB) and *R. syzygii* subsp. *syzygii*, the causative agent of Sumatra disease of clove trees in Indonesia (Roberts et al., 1990) (Remenant et al., 2011). The primers combination targeted the specific region of 16S rRNA from total genomic DNA extracted

from the symptomatic tissues of banana plants (Seal et al., 1993). Both rapid detection methods indicated the presence of pathogenic agents of BW disease of banana. However, based on the conventional method, the *R. solanacearum* or any closely related pathogenic species could not be isolated.

The morphology of most bacterial colonies (on TZC medium) isolated in this study showed similar characteristics to *R. solanacearum* as reported by Dhital et al. (2001), which were isolated from infected potatoes' stems and tubers and Ilagan et al. (2003), from banana plants infected with 'Bugtok' disease in Philippines and also Williamson et al. (2002), from leaves and stems of diseased geranium. These colonies are fluidal, irregular in shape and creamy white in colour with pink- or red-pigmented center. Based on the 16S rRNA genes amplification and sequencing, fifteen isolates which fulfilled few biochemical characteristics of *R. solanacearum* were otherwise identified as *Pseudomonas* sp. and *Stenotrophomonas maltophilia*.

On the other hand, based on the morphotyping with CHROMagar Orientation^{TM} medium, more than half of these mucoid bacterial colonies isolated from the BW-diseased banana plants were producing metallic-blue colonies. Further identification with 16S rRNA sequencing showed that the isolates were either *Enterobacter*, *Klebsiella*, *or Kosakonia* (previously known as *Enterobacter*). Isolation with the non-selective, Casamino peptone glucose (CPG) medium also indicated that majority of the isolated bacteria were members of the *Enterobacter* and *Klebsiella.* Furthermore, researchers from Malaysian Agricultural Research and Development Institute (MARDI) and Department of Agriculture (DOA), Malaysia also reported that *Enterobacter* and *Klebsiella* were the common and repeatedly isolated bacteria from BW-diseased banana plants in Malaysia (*unpublished data*). Phenotypic characteristics of those bacteria were also similar to the fluidal or mucoid morphological types of virulent, wild-type *R. solanacearum* colonies. The mucoid substance appeared around the bacterial colonies due to the accumulation of

an EPS (Álvarez et al., 2010) which is also thought to be the main factor accounting for the virulence of the pathogen (Hikichi et al., 2007).

At this stage, due to the abundance of members of *Enterobacteriaceae* from all the sampled plants in this current study, it was postulated that these endophytic bacteria could form complex biofilm structures to enhance the infection caused by the pathogenic agents of BW disease. Hence, the focus of this study was directed to understand the functions of these abundantly isolated bacterial communities in association with the BW disease of banana in Malaysia. The ability to form biofilm in plants might contribute to significant blockage of vessels in infected host plant and eventually produce the wilting symptoms (Promsai et al., 2012).

5.2 Co-inoculation of Biofilm-forming Bacteria with Blood Disease Pathogenic Strain, *Ralstonia syzygii* subspecies *celebesensis* into Banana Plants

Overall the two months' observation showed that the disease occurrence varied among all plants even in the similar treatment. It is important to note that the somaclonal variation occurred in tissue-cultured plantlets more frequently than mutations in field grown populations (Bairu et al., 2011). This *in-vitro* cultured plant materials induced or revealed variation between cells, tissues and organs due to various factors which unexpectedly creates variation within the somaclones (Emaldi et al., 2004). Based on the field observations, *Musa acuminata* cv. Berangan is less susceptible to BW disease as compared to *Musa paradisiaca* cv. Nipah (*L. Rozeita, personal communication, 2015*). The occurrence of disease-free plants in those treatments inoculated with the pathogenic strain might be due to immune responses in plant vascular system which are able to suppress the expression and colonization of the bacteria (Misas-Villamil et al., 2011). Unfortunately, due to the difficulties in propagation of tissue-cultured plantlets of banana

cv. Nipah (more susceptible to BW disease), therefore, banana plantlets from cv. Berangan was chosen as the host plants for this study.

The experimental evidence showed that treatments with monoculture inoculation of the biofilm-forming bacteria (T2, T4, T6, and T8) resulted in diseased-free plants. This data indicated that none of these tested bacterial strains served as the causative agent of BW disease. The statistical analyses also indicated that only these four treatments were significantly different with T9 (positive control). However, co-inoculation of pathogen, blood disease bacterium (BDB) with each one of these biofilm-forming bacterial strains affected the progression and severity of infections. For instance, co-inoculations of BDB with *K. radicincitans* (T5), caused rapid disease progression and more severe symptoms as compared to other treatments with the presence of BDB. This data might suggest that the co-infection of *K. radicincitans* with the pathogenic strain caused severe disease development due to synergistic interactions among those bacteria (Lamichhane & Venturi, 2015). Although *K. radicincitans* has never been reported as a phytopathogen, the whole genome analyses of the bacterium has identified a Type VI secretion system (T6SS) gene clusters in the genome of this strain (Suhaimi et al., 2014). T6SS has been suggested to be involved in conveying pathogenicity characteristics to few studied pathogenic species (Shrivastava & Mande, 2008).

DSI scores for the external symptoms (ESI) showed severe interpretation as compared to the internal symptoms (ISI) (Table 4.7). This data indicated that initially the infection process developed at the upper part of the plant where yellowing and wilting of foliage first took place. This observation could explain the characteristics of the vascular bacterial pathogen which blocked the xylem vessels and interfered directly with water movement from the plant roots to the upper part of the plant (Álvarez et al., 2010). However, for T5 plants DSI scores for the ESI and ISI were the highest (4.1 and 3.4, respectively) and interpreted as highly symptomatic. This condition explains the rapid progression of

disease symptoms and plants were highly affected due to co-infection of the inoculated bacteria (BDB and *K. radicincitans*). In contrast, DSI score for the ISI of T3 (co-inoculated with *E. cloacae* and BDB) plants was interpreted as tolerant, which means the internal parts of the inoculated plants developed slower symptoms after the infection process and were less affected by the disease.

Cross-section of infected plants showed that discoloration of internal vascular tissues was initially detected at the rhizomes, not at the primary-inoculated site. This brown discoloration of vascular tissues might occur due to the production of phenolic compound, which was activated by plant as a resistance mechanism to limit the infection (Vasse et al., 2005). This observation further suggests that following the inoculation, the bacteria probably moved down to the bottom part of the host plants, against the sap stream in the xylem vessels and colonized the vascular tissues of the rhizome before progressing to the upper part of the plant. In common infection process of *R. solanacearum* species complex, the pathogen colonizes the xylem vessels and moves throughout the stem to the upper part of the plant by just following the water conducting system (Álvarez et al., 2010).

SEM images of plants with monoculture inoculation of biofilm-forming bacteria (T2, T4 and T8) showed that the bottom part of pseudostems and rhizomes were free from bacterial cells colonizing the vascular tissues. However, the inoculated bacteria could still be isolated from the treated plants. This finding suggests that in monoculture inoculation treatments, the bacterial strains remain at the primary inoculation site. Whereas, the SEM images of diseased (symptomatic) plants tissues revealed the accumulations of bacterial agglomerates in the vascular bundles of the rhizomes. Massive accumulations of vascular pathogens caused the host plant to wilt due to clogging of the xylem vessels (Genin & Boucher, 2002). Furthermore, Muthoni et al. (2012) also explained that *R. solanacearum* is able to cause wilting due to formation of slime that surrounds the bacterial mass in the stem vascular bundles which blocked the water transport system. Most plants under T1,

T5 and positive control treatment (T9) were severely affected due to the BW infections. The SEM images demonstrated that in T1 plant, the pathogenic bacteria was not densely populated in the xylem vessels of the plant while in positive control and T5 plants, tightly-packed, adherent bacterial cells forming a developed biofilm was observed. Three-dimensional structures composed of cellular masses that are heavily enmeshed within an EPS, which develop from a foundation of adherent cells were detected in those plants. Koutsoudis et al. (2006) stated that the production of large amount of EPS is crucial in bacterial adhesion and biofilm formation of a phytopathogenic bacterium, *Pantoea stewartii* subsp. *stewartii*. This structure blocks the free-flow of water in the water-conducting vessels, leading to the wilting condition of the host plant.

On the other hand, a T3 plant which was co-inoculated with *E. cloacae* and BDB showed less bacterial colonization in xylem vessels of plant rhizome with poor EPS production. This condition might be due to the antagonistic effect of *E. cloacae* which suppressed the pathogen from multiplying and colonized the xylem vessels. A T7 (inoculated with *E. hormaechei* and BDB) plant also developed a less populated bacterial aggregates colonizing the xylem. Although the EPS was synthesized, the bacterial cells were collapsed to one side of the xylem wall, presumably due to low adhesion property (Koutsoudis et al., 2006). Kang et al. (2002) also reported that an adhesion-deficient mutant of *R. solanacearum* is less virulent and failed to form a 3D structure of bacterial aggregates. In another study, a greenhouse experiment done by Ramesh et al. (2009) had identified two antagonistic endophytes, *Enterobacter* sp. which could suppress more than 70% of the bacterial wilt incidents caused by *R. solanacearum* through the production of an antibiotic, 2,4-diacetylphloroglucinol (DAPG) which inhibited the growth of the pathogen. Barka et al. (2002) further demonstrated that simultaneous *in-vitro* co-culture of a pathogen, *Botrytis cinerea* with the antagonist shows the inability of the antagonistic bacteria to suppress the spread of pathogenic fungus but showed adverse effect when the

antagonist was introduced earlier before the pathogen. In future studies, this approach could be considered to confirm the ability of these two biofilm-forming strains (*E. cloacae* and *E. hormaechei*) to completely inhibit the bacterial wilt infections in banana plants.

This study showed that endophytic biofilm-forming bacteria in banana plants not only enhance, but also reduce the development and severity of infection caused by the blood disease pathogen, *R. syzygii* subsp. *celebesensis*. Furthermore, the SEM data evidently showed that co-inoculations of the biofilm-forming strains (*E. cloacae* and *E. hormaechei*) with the pathogen had affected the adhesion and host colonization mechanisms of the pathogenic strain. With further research, these two *Enterobacter* strains could be considered as biological control agents (BCAs) for banana bacterial wilt disease in Malaysia. A review revealed that majority of BCAs are bacteria (90%) while only 10% are fungus (Nion & Toyota, 2015). The possible suppression mechanisms of these BCAs are sustained by various interactions such as competition for nutrients and space, antibiosis, parasitism, induced systemic resistance and production of enzymes that degrade the cell wall and siderophores (Cook & Baker, 1983; Nion & Toyota, 2015). Other possible biocontrol strategies for banana bacterial wilt being implemented are, application of endogeic earthworms (Teng et al., 2016a) and the amendments of organic matter such as plant residues (Cardoso et al., 2006), animal wastes (Gorissen et al., 2004) and simple organic compounds (Posas et al., 2007).

It is well-known that some strains of *E. cloacae*, *E. hormaechei* and *K. pneumoniae* are human pathogenic bacteria (Mezzatesta et al., 2012; Siu et al., 2012; Wenger et al., 1997). However, a study of genome analysis and virulence prediction of *K. pneumoniae* 342, a nitrogen-fixing endophyte had revealed that the pathogenicity of the strain is attenuated as compared to the clinical strain, MGH78578 (Fouts et al., 2008). In addition, dissimilarities were identified in genetic contents of these two strains which influences

their preferred host ranges and lifestyles. However, in future, the virulence ability of *E. cloacae* and *E. hormaechei* strains (showing antagonistic effects on the bacterial wilt infection) isolated in this study need to be validated to better understand the potential of these endophytic bacteria in causing human or animals' disease.Synergistic and antagonistic interactions of these endophytes-pathogen resulted in more or less severe infections to the host plants (Suhaimi et al., 2016). Based on this *in-vivo* assessment, a biofilm-forming bacterium, *K. radicincitans,* which could enhance the severity of infection by the pathogenic bacteria, *R. syzygii* subsp. *celebesensis* was further selected for the whole genome analyses to further decipher the synergistic characteristics of this bacterium.

5.3 Whole Genome Sequence of *Kosakonia radicincitans,* UMEnt01/12, a Biofilm-forming Bacterium associated with Bacterial Wilt-diseased *Musa* spp.

The taxonomic evaluation of the genus *Enterobacter* by Brady et al. (2013) proposed that, the *Enterobacter radicincitans* was reclassified into *K. radicincitans* comb. nov. based on the Multilocus sequence analysis (MLSA) group. The maximum likelihood tree of 16S rRNA gene sequences of members of *Enterobacteriaceae* indicated that *Kosakonia* spp. are clustered together with *E. cloacae* and *Citrobacter youngae* (Brady et al., 2013). Analysis of the whole genome of this *K. radicincitans* UMEnt01/12, a biofilm-forming endophytic bacteria which was isolated from the pseudostem of BW-diseased banana plant revealed several features of this bacterium: the survivability, biofilm-forming ability and the endophytic colonization ability of the bacterium in the endosphere of the banana plants. In particular, the presence of large arrays of genes related to pathogenicity such as the toxin-antitoxin, Type IV and Type VI secretion proteins, biofilm formation and other

genes related to fitness advantages suggested that the bacterium maybe pathogenic in nature although the extent of its pathogenicity remains unclear.

Interestingly, the genome of *K. radicincitans* UMEnt01/12 encodes two toxin/anti-toxin (T/A) systems (*CcdA* and *RelE*) which could play biological roles in stress resistance (Aizenman et al., 1996), protection from bacteriophages (Fineran et al., 2009), and regulation of biofilm formation (Ghafourian et al., 2013; Wang & Wood, 2011) indicating that the bacterium habour the pathogenic potential to infect the host. T/A systems are closely related to pathogenicity, where the presence in the genome may facilitate the transfer of pathogenic genes via horizontal gene transfer such as through mobile genetic elements or plasmid mediated. Furthermore, the presence of the T/A biofilm protein, HipA in the genome is found to play significant role in enhancing biofilm formation through DNA release (Zhao et al., 2013). The small number of T/A systems in the genome represent this bacterial strain as the host-associated microorganism (Pandey & Gerdes, 2005).

Endophytic microorganisms commonly share resources with their associated host plants (Taghavi et al., 2010). Therefore as expected, the genome of *K. radicincitans* UMEnt01/12 codes for a large diversity of transporters that will allow for the uptake of plant-produced nutrients (638 ORFs encode for transporter genes). It has been shown that other genomes of plant/ soil-associated bacteria also code for high number of transporters such as *Bradyrhizobium japonicum* (986 transporter genes), *Mesorhizobium loti* (885), and *Agrobacterium tumefaciens* (835), also the plant pathogen, *Erwinia carotovora* (631) (Ren & Paulsen, 2005; Ren & Paulsen, 2007; Taghavi et al., 2010). The UMEnt01/12 genome encodes 38 phosphotransferase system (PTS) transporters which is almost the same as *Enterobacter* sp. 638 (41 PTS transporters) (Taghavi et al., 2010). The high number of PTS genes in this bacterial genome is expected as it was reported that *Enterobacteriales* (such as *E. carotovora* subsp. *atroseptica* SCRI1043 and *E. coli*

CFT073) is having higher number of PTS genes as compared to other members of Gamma-Proteobacteria subdivision (e.g. *Pseudomonadales* and *Xanthomonadales*) (Barabote & Saier, 2005).

Also, *K. radicincitans* UMEnt01/12 is possibly motile inside its host plant's tissues, since endophytes tend to colonize specific plant parts that do not always correspond to the port of entry in the plant (Taghavi et al., 2010). The genome contains various genes encoding for proteins related to flagellar biosynthesis and transcriptional regulators that are relevant to motility. Those proteins are also found in other endophytic bacteria such as *Enterobacter* sp. 638, *Salmonella enterica* and *Escherichia coli* K-12 (Taghavi et al., 2010). Genes encoding for adhesion proteins found in the bacterial wilt phytopathogen, *R. solanacearum* were also detected in the genome of UMEnt01/12 strain, reported as the candidate genes involved in pathogenesis of *R. solanacearum* strain (Salanoubat et al., 2002). Proteinaceous structures such as pili, outer membrane proteins, and lipopolysaccharides (LPSs) have been reported to affect adhesion among the colonizing bacteria in plant tissues (Bogino et al., 2013). Pili (fimbriae) serve as adhesive-like component to bind the cells in both plant–bacterium and bacterium–bacterium interactions during the colonization (Simpson et al., 2000). This idea was supported by the scanning electron microscopy (SEM) images of plant which was inoculated with this UMEnt01/12 strain (shown in Figure 4.27c), showing bacterial clumps detected in the rhizome tissues, far from the inoculation site.

Besides, the LPS are also indicated in other earlier studies to be involved in cell motility, biofilm formation, and contributes substantively to antibiotic resistance (Czyzyk et al., 2011). The LPS inner-core OS in *K. radicincitans* UMEnt01/12 strain composed of three heptosyltransferase (HepI, HepII, and HepIII), also found in endophytic bacteria, *E. coli* and *Salmonella.* HepI transferase was reported to be involved in the transfer of Hep to a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) of the growing core region of LPS. The

other two heptosyltransferases are known to transfer Hep to Re-LPS and Rd(2)-LPS, respectively (Nakao et al., 2012). The bacterial biofilm can be of a single species, while in natural environment the formation involved many bacterial species (Rafique et al., 2015). When the diversified bacterial communities occupied the plant tissue surfaces, the EPS production make their adhesion irreversible (Meneses et al., 2011; Rafique et al., 2015). These bacteria use signal molecules, referred to as the autoinducers, to monitor their population density and coordinate gene expression in a process named quorum-sensing (Nasser & Reverchon, 2007). The quorum-sensing (QS) was first described in the LuxI/LuxR system of a symbiotic species, *Vibrio fisheri* (Nealson & Hastings, 1979). The acylated homoserine lactones (AHLs) are the most common autoinducers for cell-to-cell communication in Gram-negative bacteria which has been found to influence the biofilm formation (Kumar et al., 2016; Xu et al., 2015). When AHLs reached a threshold concentration indicating higher bacterial population density, these signals interact with LuxR transcriptional regulators to initiate specific genes expression (Dong et al., 2001). Presence of LuxR family proteins in the UMEnt01/12 strain probably serve as regulators of the AHLs synthesized by other colonizing bacteria, specifically the pathogenic strain, *Ralstonia solanacearum* in which contribute towards the formation of complex biofilm structures in the host plant. The pathogenic bacterium produces 3-hydroxypalmitic acid methyl ester as a novel signalling molecule, which together with AHLs, are used to regulate virulence in the host plants (De Kievit & Iglewski, 2000; Flavier et al., 1997). Taken together, the presence of these motility and biofilm related genes indicate that the organism is highly motile and potentially capable of colonizing deep tissues of the host and causing none to mild pathological symptoms, depending on its synergistic effect with other bacteria as well the plant's immune responses. However, it is apparent that the interaction between the host and the bacterium is more complex than initially thought due

to its complex gene repertoires and gene-gene interactions that resembles partly, if not all of other well-known plant pathogens.

As *K. radicincitans* UMEnt01/12 belongs to the *Enterobacteriaceae,* a family that consist of both beneficial plant associated bacteria as well as opportunistic pathogens such as the dualistic life style organisms *Klebsiella pneumoniae* and *Enterobacter cloacae*, both of which can be regarded as endophytes (Fouts et al., 2008; Shankar et al., 2012) and the opportunistic pathogens (Schwartz & Otto, 2000; Wu et al., 2009). Hence, the organism is expected to share some of their bacterium-host association strategy, where such observation is apparent especially with respect to their commonly found gene contents. For instance, the genome analysis revealed that UMEnt01/12 strain possessed a Type VI secretion system (T6SS) clusters (Hcp1, Vgr, EvpB, DotU, and ImpA) in its genome. T6SS is a system that composed of 15–20 proteins, conserved in both pathogenic and non-pathogenic bacteria whose functions are not fully understood (Boyer et al., 2009; Filloux et al., 2008; Sarris et al., 2010; Shrivastava & Mande, 2008). The T6SSs are widespread among Proteobacteria, suggesting that they may be involved in yet unknown pathogenic or symbiotic lifestyles or other types of cell-to-cell communications (Sarris et al., 2010). Earlier studies have shown that T6SS contributes to the virulence development of various pathogens such as *Pseudomonas aeruginosa* (Mougous et al., 2006), enteroaggregative *E. coli* (Dudley et al., 2006) and *Vibrio cholerae* (Pukatzki et al., 2006), also some plant pathogenic bacteria, including *Agrobacterium tumefaciens* (Wu et al., 2008) and *Pectobacterium atrosepticum* (Liu et al., 2008). *K. radicincitans* UMEnt01/12 strain contains Hcp1 and Vgr family proteins which required for the secretion of each other, suggesting that both might be part of the secretion apparatus (Jani & Cotter, 2010). The Hcp proteins secreted in T6SS-dependent manner is most significantly demonstrated in *Vibrio cholerae* which comprises of two *hcp* genes (*hcpA, hcpB or hcp1, hcp2*) encoding for the identical proteins, Hcp1, -2 (Filloux et al., 2008; Pukatzki et al., 2006).

Whereas, the *vgr* T6SS genes together with *hcp-2* and *clpV* was found in *Citrobacter freundii* which regulate flagellar system and enhance motility for the adherence of bacterium and induces cytotoxicity to host cells (Liu et al., 2015). It is demonstrated that the T6SS-dependent secretion of at least one Hcp protein is required and sufficient for virulence (Filloux et al., 2008).

In addition to pathogenesis, T6SS may also modulate root colonization/nodule formation through symbiotic interactions of the nitrogen-fixing bacteria such as, *Mesorhizobium loti*, *Rhizobium leguminosarum* (Bingle et al., 2008) and *Cupriavidus taiwanensis*, which belongs to the phylogenetically distinct β-Rhizobium group (Amadou et al., 2008). Boyer et al. (2009) further proposed that the T6SS has evolved to assist the bacteria in adaptation to various microenvironments and specialized functions. Later analyses of prokaryotic genomes have revealed that these versatile secretion systems may function in promoting commensal or mutualistic relationships between bacteria or facilitating the interactions between bacteria and may also be used for the intraspecies microbial cooperation (Jani & Cotter, 2010; Konovalova et al., 2010). Considering all the possible functions of this T6SS in different bacterial species, the expressions of this system in UMEnt01/12 strain have yet to be explored.

K. radicincitans was never reported as a putative phytopathogenic bacteria and yet so far, only as biological nitrogen-fixing endophytic bacteria with growth-promoting effects (Kampfer et al., 2005; Witzel et al., 2012). However, the genome comparison between the UMEnt01/12 and the DSM 16656 strains revealed some dissimilarities in the distribution of genes related to Type IV and Type VI secretory functions which may impact secretions of pathogenesis-related factors or substances that promote the associations of UMEnt01/12 strain with BW disease in banana plants. The Type II secretion system (T2SS) which is essential for pathogenicity of *R. solanacearum* (Kang et al., 1994) was also found in the UMEnt01/12 genome. In this pathogenic bacterium,

the plant cell wall degrading enzyme are secreted through the T2SS. Meanwhile, Type IV secretion proteins, Rhs found in UMEnt01/12 genome could be related to virulence properties as identified in *P. aeroginosa* (Koskiniemi et al., 2013) or might be facilitating in the intercellular competition among diverse bacteria as reported by Kung et al. (2012).

Despite the presence of other genes related to pathogenicity, the UMEnt01/12 genome lack of a type III (Hrp) secretory system, which is considered as a prerequisite characteristics of active virulent life style for plant pathogenic bacteria in the genera *Erwinia, Pseudomonas, Xanthomonas,* and *Ralstonia* (Alfano & Collmer, 1997), suggesting that the *K. radicincitans* may utilize unknown and yet-to-characterized pathogenicity mechanism in its interaction with the host, potentially via host cell modulation or other form of synergistic interaction. This notion is supported by the observation from the glasshouse experiment, in which the monoculture inoculation of (Treatment 6) into the banana plants resulted in non-diseased plant but symptoms of burnt young shoots were instead observed in some of T6 plants that indicate the affected plant tissues (shown in Figure 4.15a and 4.27b). Hence, it is reasonable to suggest that the presence of this bacterium alone is not sufficient to induce severe pathological symptoms but could potentially interact synergistically with other agents of BW disease to develop rapid disease progression and cause more severe infection as indicated in the co-inoculation study. It is yet challenging to determine the thresholds and co-infection factors considering the multiple factors are in place compounded with complex bacterial-host interaction. However, it is of interest to establish this interesting link that warrants further and large-scale investigation in the future study.

The whole genome analysis of *K. radicincitans* UMEnt01/12 discovered the potential mechanisms of interaction that result in communication and synergism of this bacterium with other causative agent such as the banana blood disease pathogen, *R. syzygii* subsp. *celebesensis.* The presence of genes related to biofilm formation, cell-to-cell

communications (quorum sensing), potential pathogenesis and various secretion systems reflects this microbe's ability in enhancing the development and severity of bacterial wilt infections. In summary, the culture-dependent technique in studying bacteria associated with BW-diseased banana plants in Malaysia, indicated that various bacterial genera could be isolated from the symptomatic and non-symptomatic BW-diseased banana plants. This study also revealed that those endophytic bacteria have potential antagonistic and synergistic effects with the pathogens in the occurrence and severity of the infections. Hence, a culture-independent technique will provide a deeper insight into the complex communities of the culturable and non-culturable microbiota which associated with the BW-diseased banana plants.

5.4 Diversity of Bacterial Communities associated with Symptomatic and Non-symptomatic Bacterial Wilt-diseased Banana Plants

Little information is available concerning the diverse consortium of plant-associated microbiota in banana plants (Koberl et al., 2015). The 16S rRNA metagenomics sequencing and analysis is the most powerful approach to reveal the diversity and abundance of microbial communities that reside in the endosphere of banana plants with and without (might be latent infection) BW-diseased symptoms. The characterization of the bacterial population using 16S rRNA PCR amplification of the highly variable, V3 region of the 16S rRNA gene was chosen in this study because it has been shown that the bacterial community analysis and data quality has been greatly improved in this region (Bartram et al., 2011).

Commonly in plants, the rhizosphere is a hotbed of microbial activity (Herron et al., 2014), which is characterized by the high microbiome abundance and diversity (Berendsen et al., 2012; Berg et al., 2015). Only a portion of this root-colonizers is able

to invade, compete with other well-adapted endophytes, and successfully colonize the inner plant tissue (Chi et al., 2005). However, in banana plants, the succulent pseudostem (endosphere) has previously been reported to host an extraordinary high abundant and diverse microbial community as compared to other microenvironments of the plant (Rossmann et al., 2012). Thomas and Sekhar (2014) had also revealed the widespread prevalence of endophytic bacteria in banana with majority of non-culturable type. Koberl et al. (2015) stated that, the high abundance and diversity of the endophytes in the banana plant can be explained by the permanent nature of its corm serving as a reservoir for endophytic diversity and the transmission to following generations via vegetative suckers. Therefore, this microenvironment is the most suitable plant part to represent the microbial populations in banana plants.

Prior to the metagenome sequencing, procedures for the extraction of pure and high quality genomic DNA from banana tissue samples is a crucial prerequisite step. However, secondary metabolites, polysaccharides and polyphenolics compounds produced by plants may interfere with the extraction of high quality DNA and subsequent molecular applications (Sika et al., 2015). Banana (*Musa species*) plants contain high amounts of polyphenols and polysaccharides level, also secondary metabolites such as alkaloids, flavonoids and phenols (Das et al., 2009; Siddique, 2014). Hence, the CTAB-based extraction method was reported to be efficient in eliminating PCR inhibitors from the extracted DNA (Rahman et al., 2010). However in this study, based on the Nanodrop™ spectrophotometer reading (average A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.5 – 2.0 and < 0.5, respectively), the A_{260}/A_{230} ratios were still far below 2.0, which is a required value of purity for the DNA (Desjardins & Conklin, 2010). Hence, polysaccharides contamination might be still abundant in the DNA samples extracted using this DNA extraction protocol. This result indicated that the first protocol failed to totally remove the polyphenolics and polysaccharides from the banana pseudostem samples.

In contrast, the second protocol which was modified from Fan and Gulley (2001) and Souza et al. (2012) successfully produced high quality genomic DNA (average A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.8 – 2.0 and > 1.8, respectively). This modified method initially utilizes sorbitol buffer to remove the sticky polysaccharides substance (Souza et al., 2012). Sodium dodecyl sulphate (SDS) in the extraction buffer lysed the cell membranes and nuclei to release the DNA (Sika et al., 2015). Furthermore, this DNA isolation protocol eliminate the use of phenol and chloroform which are known as toxic, hazardous and expensive organic reagents (Niu et al., 2008). Hence, this DNA extraction method was chosen as the most suitable, less hazardous and effective for downstream 16S rRNA metagenomics analysis.

Based on the taxonomic classification (Table 4.12), Cyanobacteria and Proteobacteria accounted for over 99% of total bacterial populations identified in the non-symptomatic (NS) and two symptomatic (SA and SB) banana plant samples. Members of Cyanobacteria are able to form symbiotic associations with a wide variety of plants (Adams & Duggan, 2012) which might contribute to the strong interaction with their host. The Proteobacteria is well-recognized as the most abundant phylum present in plant tissues in the domain Bacteria (Ettema & Andersson, 2009). Plant pathogens are also mostly confined to the Gram-negative Proteobacteria. The most studied phytopathogen was characterized under the class of alpha (α)-, beta (β)-, and gamma (γ)-Proteobacteria (Mansfield et al., 2012).

At the family level, the three leading families found in all the three plant samples were Burkholderiaceae, Methylobacteriaceae and Sphingomonadaceae. This result was in contrast with that reported by Rossmann et al. (2012), where Enterobacteriaceae was found to be highly abundant in the pseudostem of East African Highland banana plants from three different traditionally cultivated (amendments of human and animals manure) farms in Uganda. Another recent study by Koberl et al. (2015), which focused on γ-

Proteobacteria communities in different microenvironments (biogeography and agroforestry) of banana plants from farms in two different countries, Nicaragua and Costa Rica, in Central America, and from sites with and without associated *Fabaceae* trees, also showed that three dominant families are Pseudomonadaceae, Moraxellaceae and also Enterobacteriaceae. This disparity was probably due to conditions of the plants which were infected by the BW disease. This is because of the high abundance of members of Burkholderiaceae in all the three plant samples, which were dominated by *Ralstonia,* a genus which belongs to the pathogenic agents of BW disease of banana (*R. solanacearum* and *R. syzygii*). Whereas, it was reported that the members of Methylobacteriaceae and Sphingomonadaceae are commonly found as the endophytic bacteria in several plants due to their unique ability to utilize methanol discharged by wall-associated pectin metabolism of plant cells (Pini et al., 2012).

At the genus level, *Ralstonia* was abundantly identified in those symptomatic and non-symptomatic plants. Although not all species under this genus were reported as phytopathogens, the main causative agents of banana BW disease in Malaysia are *R. solanacearum* (Zulperi & Sijam, 2015) and *R. syzygii* subsp. *celebesensis* (Teng et al., 2016b). These two plant pathogenic species might be abundant in those plant samples, however due to the close similarities of the 16S rRNA gene sequences of *Ralstonia* sp., they were indistinguishable at the lowest, species level (based on the NCBI 16S microbial database). Furthermore, serological detection with ImmunoStrip test and PCR amplification and sequencing which target the 16S rRNA species-specific region showed positive results of the presence of *Ralstonia* species complex in all the samples. Hence, those symptomatic and non-symptomatic banana plants could possibly harboured these pathogenic bacterial species.

Meanwhile, the non-rooted relationship between the three studied plants (NS, SA and SB) depicted that, although non-symptomatic (NS) and symptomatic A (SA) plants were

collected from the same geographical location, the microbiota of NS plant were distinctly different from those in the two symptomatic (SA and SB) plants. Koberl et al. (2015) proposed that biogeographical location and agroforestry conditions led to shift within the individual taxonomic groups of γ-Proteobacterial communities populating the sampled banana plants. However, this present study indicated that, diseased host plants had higher impact on the total microbial communities residing in the plants than the biogeographical location. Turner et al. (2013) had stated that minor changes in the host genome can directly influence the microbiota of the plants, and these changes can feed back to modify the behaviour of the host.

The 16S rRNA metagenomics sequencing indicated that the *Ralstonia* spp. was abundant in the diseased plant samples, yet the earlier conventional method failed to isolate these bacteria. This flaw might be due to the overgrowth of fast-growing endophytes which also had similar morphologies on the isolation media that led to the false-negative results. Besides that, the failure of *Ralstonia* species-complex to compete with many other microorganisms on the semi-selective media hampered the possibilities of isolating the desired pathogenic agents of banana BW disease (Pradhanang et al., 2000). In order to overcome the problem, increasing the incubation period for the isolated colonies on the semi-selective medium (SMSA) might improve the chances of getting the pathogens (*Lum KY., personal communication, 2013*). Plant Pathology Unit from DOA and MARDI, Malaysia had also concluded that most of the BW disease incidences of banana in Malaysia from 2009 to the present days, were caused by the *R. syzygii* subsp. *celebesensis* (BDB) (Kogeethavani et al., 2013; Timin et al., 2014) and not the *R. solanacearum*. Unlike *R. solanacearum* (pathogenic on other solanaceous plants), the morphology of BDB colonies on TZC agar is much smaller and non-fluidal (Hayward, 1992; Teng et al., 2016a). Hence in this current study, the unexpected morphology of the BDB colonies had also caused the failure in isolating the bacterium from the sampled banana plants.

In general, the 16S rRNA metagenomics analyses had revealed higher diversity and abundance of banana-associated bacteria in the non-symptomatic plant than those in the symptomatic plants. As suggested by Reiter et al. (2002), abundance of genera in non-symptomatic (infected but showing no diseased symptoms)plant might be influenced by the availability of nutrients and metabolites in this uninfected plant, allowing favourable conditions for the growth of more diverse endophytic bacteria. Reiter and co-workers (2002) also proposed that the endophytic bacterial communities in the non-symptomatic plant could be associated with pathogen defence mechanisms of plant which contribute towards maintaining the pathogen population lower than the level required for disease to occur.

In contrast, the diversity and abundance of the endophytic communities associated with the symptomatic plants were noticeably lower as compared to the non-symptomatic plant. This might be due to the competition for nutrient sources and favourable niches with the pathogenic bacteria which led to a reduction of species richness in the infected plants (Bulgari et al., 2011). However, based on this present analyses, varying abundance of the diverse bacterial populations in the symptomatic and non-symptomatic banana plants could not possibly determine the contribution of the microbiota towards different plant physiological status or the plant conditions (diseased and non-diseased).

CHAPTER 6: CONCLUSION

Since the outbreak of bacterial wilt (BW) disease of banana in Malaysia, many researchers have been trying to identify the pathogenic bacteria associated with the disease. The first report had indicated that the causative agent of banana BW disease in Malaysia was identified as *Ralstonia solanacearum.* However in recent years, there were discrepancies in the identification and characterization studies of the causative agents such as *R. solanacearum* phylotype II, *R. solanacearum* phylotype IV and *R. syzygii* subsp. *celebesensis* (blood disease bacterium). This present study failed to isolate the pathogenic bacterium of banana BW disease from all the collected plant samples. However, majority of the isolated bacteria which associate with the disease were identified as the members of *Enterobacteriaceae*. Hence, this study for the first time focused on the abundant communities of the non-pathogenic bacteria isolated from infected banana plants in order to understand the involvement of these bacteria in the occurrence and progression of BW-diseased in their host plants.

Further characterization based on biofilm-forming ability of the isolated bacterial strains indicated that most of the tested strains were strong biofilm-formers. The mono-culture inoculation study demonstrated that the four selected strong biofilm-formers (*Klebsiella pneumonia, Kosakonia radicincitans Enterobacter cloacae* and *E. hormaechei*) could not cause the banana BW disease. However, in the co-culture inoculation, those four strains revealed potential antagonistic and synergistic effects towards the development of the disease with the presence of pathogenic strain, blood disease bacterium (BDB). In addition, the scanning electron microscopic analysis evidently showed that two biofilm-forming strains (*E. cloacae* and *E. hormaechei*) hampered the adhesion and host colonization mechanisms of the pathogen (BDB). Conversely, the other two strains (*K.*

pneumoniae and *K. radicincitans*) have facilitated and enhance the colonization of BDB in vascular tissues of the inoculated plants. Genome annotation and analyses of a strong biofilm-former, *K. radicincitans* which had enhanced the infection and colonization by the pathogenic bacterium (BDB) has provided a deeper understanding of the synergistic lifestyle of this bacterium through biofilm formation ability, quorum-sensing mechanism, potential pathogenesis and various secretion systems. Finally, the 16S rRNA metagenomics sequencing and analyses have revealed that the bacterial population in the non-symptomatic plant (harbour the pathogenic bacteria but was not producing symptoms) are comparatively abundant and richer in biodiversity as compared to those in the symptomatic plant (infected and produced BW symptoms).

In the light of success summarized above, the following approaches could be undertaken to further advance the research:

1. To determine whether the two biofilm-forming strains (*E. cloacae* and *E. hormaechei*) which were suspected as antagonists play a role as protectant or curative or combination of both in controlling the bacterial wilt disease of banana.
2. To conduct a glasshouse experiment where the antagonist will be inoculated into the plants earlier before the pathogenic bacterium in order to identify the ability of the antagonistic bacteria to completely inhibit infection in the host plant.
3. To compare the genome sequence of *Kosakonia radicincitans* UMEnt01/12 with other plant-associated bacteria which have the ability to become potential pathogenic agent in favourable conditions.
4. To identify the metabolic functions of microbial population dynamics and the mechanisms occurring in the healthy (disease-free), symptomatic (infected with

symptoms) and non-symptomatic (infected without symptoms) bacterial wilt-diseased banana plants.

5. To discover whether the more diverse bacterial population found in the non-symptomatic plant responsible for the antagonistic effects and control of the pathogenic bacteria in their host plant.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

The list of research papers published and presented in the conferences and seminars.

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Suhaimi, N. S. M., Yap, K. P., Ajam, N., & Thong, K. L. (2014). Genome sequence of Kosakonia radicincitans UMEnt01/12, a bacterium associated with bacterial wilt diseased banana plant. *FEMS microbiology letters*, *358*(1), 11-13.

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