Mircobacterium foliorum STRAIN SZ1 ASSISTED *Melastoma malabathricum* L. PHYTOREMEDIATION OF ARSENIC

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DEDICATION

This thesis is dedicated to my beloved parents, who have been my source of support and encouragement throughout the study.

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ABSTRACT

Arsenic contamination in soil is a serious problem as the toxic metalloid impacts both environmental and public health. Phytoremediation is an environmental friendly method that can be applied to remediate the arsenic-contaminated soil. Arsenic is a non-essential element and it is generally toxic to plants. The efficiency of plant arsenic uptake is usually low as most soils contain inorganic arsenic in the form of stable arsenate. Bacterial association with the plant can improve plant growth and arsenic uptake. Therefore, this project aimed to investigate the efficiency of a local plant *Melastoma malabathricum* L. incorporated with an arsenate-reducing bacterium *Microbacterium foliorum* strain SZ1 in arsenic phytoremediation. The effects of *M. foliorum* SZ1 inoculation on the soil bacterial community and the arsenic-exposed *M. malabathricum* L. leaf proteins expression were also studied. A two-month experiment was conducted at the greenhouse using *M. malabathricum* L. treated with four arsenic concentrations (0, 10, 30 and 50 ppm), inoculated or uninoculated with *M. foliorum* SZ1. Plant dry weight, root length and shoot length were measured as growth assessment. Arsenic contents in soil and plant tissues (roots and shoots) were quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES). Illumina MiSeq 16S rRNA was applied to determine the total soil bacterial composition. Leaf proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by liquid chromatography-mass spectrometry (LC-MS). At the end of the treatment, plant's survival was only observed in the uninoculated 0 ppm treatment and all 50 ppm treatments. The 50 ppm-treated plants showed a significantly higher plant dry weight than the respective inoculated 0, 10 and 30 ppm plants by 115%, 80%, and 77%. Soil arsenic concentration of the inoculated 30 ppm treatment was 7.7% and 23.6% higher than the uninoculated 50 ppm and inoculated 50 ppm soils, respectively. The bioconcentration factor of *M. malabathricum* L. in 50 ppm arsenic was more than 1, suggesting the plant's ability in arsenic phytoextraction. Establishment of *M. foliorum* SZ1 in soil was not observed. *M. foliorum* SZ1 may have increased the abundance of its order *Micrococcales* in the 50 ppm arsenic-contaminated soil. However, the effect of inoculation on the soil was not as prominent as the arsenic toxicity. All survived plants expressed proteins that mainly involved in cellular respiration and energy metabolism. Arsenic treatment increased the leaf protein expression by almost 3-fold. Plant defense against the toxicity was determined by the discovered ROS-scavenging enzymes such as peroxidases, glutathione-S-transferase, 2-cysteine peroxiredoxin and catalase. This project assesses the efficiency of local plant-bacteria association in remediating arsenic-contaminated soil and provides information on the soil indigenous community and plant physiology. The presented data can be used as a reference to optimize the application of bacteria-assisted phytoremediation technique in future.

ABSTRAK

Pencemaran arsenik pada tanah merupakan masalah yang serius kerana metaloid toksik ini mampu menjejaskan alam sekitar dan kesihatan awam. Fitopemulihan merupakan kaedah yang mesra alam dan boleh digunakan untuk merawat tanah tercemar arsenik. Arsenik ialah elemen tidak perlu dan beracun. Kecekapan tumbuhan dalam penyerapan arsenik adalah rendah memandangkan kebanyakan tanah mengandungi arsenik tak organik dalam bentuk arsenat yang stabil. Penyekutuan bakteria dengan tumbuhan mampu meningkatkan kadar pertumbuhan dan pengambilan arsenik. Justeru, tujuan projek ini adalah untuk menyelidik kecekapan penyekutuan pokok tempatan *Melastoma malabathricum* L. dan bakteria penurunan arsenat, *Microbacterium foliorum* SZ1 dalam fitopemulihan arsenik. Seterusnya, projek ini mengkaji kesan inokulasi *M. foliorum* SZ1 terhadap komuniti bakteria tanah dan pengekspresan protein dalam daun *M. malabathricum* L. yang terdedah kepada arsenik. Kajian telah dijalankan dalam rumah hijau selama dua bulan dengan *M. malabathricum* L. yang dirawat pada 4 kepekatan arsenik (0, 10, 30 dan 50 ppm) dan inokulasi *M. foliorum* SZ1. Berat kering pokok, ukuran pucuk dan akar telah ditentukan sebagai penilaian tumbesaran pokok. Kuantiti kandungan arsenik dalam tanah dan tisu pokok (pucuk dan akar) telah dinyatakan menggunakan spektroskopi pelepasan optik plasma (ICP-OES). Illumina MiSeq 16S rRNA digunakan untuk mengkaji komposisi keseluruhan bakteria dalam tanah. Protein daun telah dipisahkan oleh sodium dodesil sulfate-gel elektroforesis poliakrilamida (SDS-PAGE) dan dikenal pasti oleh kromatografi cecair-spektrometri jisim (LC-MS). Keputusan menunjukkan kelangsungan hidup pokok bagi rawatan 0 ppm (tanpa inokulasi) dan semua rawatan arsenik 50 ppm. Berat kering pokok dirawat 50 ppm arsenik nyata sekali ganda lebih daripada pokok dirawat 0, 10 dan 30 ppm (diinokulasi) sebanyak 115%, 80% dan 77%. Kepekatan arsenik dalam tanah rawatan 30 ppm pula nyata sekali ganda lebih daripada tanah dirawat 50 ppm arsenik (tanpa inokulasi) sebanyak 7.7% dan 50 ppm (diinokulasi) sebanyak 23.6%. Faktor biokonsentrasi *M. malabathricum* L. yang dirawat 50 ppm arsenik adalah lebih daripada 1 dan kemampuan pokok dalam fitoekstraksi telah dicadangkan. Pembentukan *M. foliorum* SZ1 dalam tanah tidak dapat diperhatikan. *M. foliorum* SZ1 mungkin telah meningkatkan kelimpahan ordernya *Micrococcales* dalam tanah rawatan 50 ppm arsenik. Namun, kesan inokulasi pada tanah adalah tidak ketara seperti kesan ketoksikan arsenik. Semua pokok yang masih hidup mengekspresikan protein terutamanya melibatkan dalam proses pernafasan selular dan metabolisma tenaga. Rawatan arsenik telah meningkatkan pengekspresan protein daun sebanyak tiga kali ganda. Kemampuan pokok dalam toleransi arsenik telah didapati dengan penemuan enzim terlibat dalam peneutralan kesan spesis oksigen reaktif seperti peroksidase, glutation-S-transferase, 2-sistein-peroksiredoksin dan katalase. Projek ini telah menilai kecekapan gabungan pokok tempatan dengan bakteria untuk merawat tanah tercemar arsenik dan memberikan maklumat tentang komuniti asli bakteria dan fisiologi tumbuhan. Data yang ditunjukkan boleh digunakan sebagai rujukan untuk mengoptimumkan aplikasi teknik fitopemulihan dibantu bakteria.

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1D	-	1-dimensional
2-Cys-Prx	-	2-cysteine peroxiredoxin
2-ME	-	2-mercaptoethanol
ABC	-	Ammonium bicarbonate
ACC deaminase	-	1-aminocyclopropane-1-carboxylate
ACE	-	Abundance-based coverage estimators
ACN	-	Acetonitrile
ADP	-	Adenosine diphosphate
$AgNO_3$	-	Silver nitrate
AMOVA	-	Analysis of molecular variance
ANOVA	-	Analysis of variance
APX	-	Ascorbate peroxidase
As(III)	-	Arsenite
As(V)	-	Arsenate
ATP	-	Adenosine triphosphate
BCA	-	Bicinchoninic acid
BCF	-	Bioconcentration factor
BSA	-	Bovine serum albumin
CAT	-	Catalase
cfu	-	Colony forming unit
cm	-	Centimetre
CO_2	-	Carbon dioxide
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminatetraacetic acid
FA	-	Formic acid
g	-	Gram
GPX	-	Glutathione peoxidase
GR	-	Glutathione reductase

GSH	-	Glutathione
GSSG	-	Glutathione disulphide
GST	-	Glutathione-S-transferase
H_2O_2	-	Hydrogen peroxide
HCl	-	Hydrochloric acid
HNO_3	-	Nitric acid
Hz	-	Hertz
IAA	-	Indole-3-acetic acid
ICP-OES	-	Inductively coupled plasma-optical emission spectrometry
kb	-	Kilobases
kDa	-	Kilodalton
kg	-	Kilogram
L	-	Litre
LB	-	Luria-Bertani
LC-MS	-	Liquid chromatography-mass spectrometry
LSU	-	Large ribosomal subunit
M	-	Mol
mg	-	Milligram
Min	-	Minutes
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimoles
m/z	-	Mass-to-charge ratio
NaCl	-	Sodium chloride
NADPH	-	Nicotinamide adenine dinucleotide phosphate hydrogen
ng	-	Nanogram
NGS	-	Next generation sequencing
nL	-	Nanolitre
nm	-	Nanometre
OUT	-	Operational taxonomic unit
PC	-	Principle coordinate
PCs	-	Phytochelatins
PCoA	-	Principal coordinate analysis

PGPB	-	Plant growth-promoting bacteria
ppb	-	Parts per billion
ppm	-	Parts per million
rcf	-	Relative centrifugal force
ROS	-	Reactive oxygen species
RuBisCO	-	Ribulose-1,5-biphosphate carboxylase/oxygenase
SDS-PAGE	-	Sodium docedyl sulfate-polyacrylamide gel electrophoresis
SOD	-	Superoxide dismutase
SSU	-	Small ribosomal subunit
TAE	-	Tris-acetate-EDTA
TEMED	-	Tetramethylethylenediamine
TF	-	Translocation factor
Tris	-	Trisaminomethane
UV-Vis	-	Ultraviolet-visible
v/v	-	Volume per volume
w/v	-	Weight per volume
µg	-	Microgram
µL	-	Microlitre
µM	-	Micromole
µm	-	Micrometre

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Arsenic is known as a toxic metalloid to all forms of life. It ranks first in the Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List due to its high frequency, toxicity and potential for human exposure (ATSDR, 2019). Excessive uptake of arsenic by living organisms usually lead to poisoning as it is a non-biologically essential element. Both bioaccumulation and biotransformation of arsenic induce oxidative stress in the cellular systems by creating imbalances between reactive oxygen species (ROS) and antioxidants (Jomova et al., 2011). The equilibrium between ROS production and scavenging determines whether they would serve as signaling molecules or cause cellular oxidative inflammation (Venditti, Napolitano and Di Meo, 2015). Prolonged cellular damage by ROS implicates in the pathogenesis of cancer, cardiovascular diseases and possibly diabetes (Flora and Agrawal, 2017).

Being the 20th most abundant element in the Earth's crust, arsenic can be introduced into the environment in natural and anthropogenic ways. Generally, the metalloid presents an organic form and an inorganic form with oxidation states of +3 and +5 (Mandal and Suzuki, 2002). Lakes, rivers, and groundwater contaminations of arsenic are the consequences of its solubility in water. The utilization of the contaminated water for irrigation and consumption cause arsenic exposure to living organisms. Due to the non-degradable state of arsenic, it is cycled through all environmental compartments continuously. The source of arsenic in the soil and water impacts the ecosystem health and reduce the available environmental resources (Chung, Yu and Hong, 2014).

Phytoremediation is a plant-based technology that utilizes plants' natural abilities to transform and accumulate heavy metals in the surrounding soil and water environments. Several soil phytoremediation techniques such as phytoextraction, phytostabilization and phytovolatilization facilitate the contaminant removal (Ali, Khan and Anwar, 2013). A previous review drew the interest of the heavy metal remediation using plant-bacteria association (Glick, 2010). The integrated term bacteria-assisted phytoremediation (or phytobial-remediation) defines the involvement of bacteria with specific mechanisms such as facilitating the plant metal uptake and enhancing the plant vigor. Beneficial bacteria can have a synergistic relationship with the plant via three methods, including the attachment at the rhizosphere (rhizospheric), forming nodules on plant roots (nitrogen-fixing symbiotic) or colonizing in plant's interior tissues (endophytic) (Glick, 2012). The multidiscipline bacteria-assisted phytoremediation technique also applies to soil arsenic mitigation (Ullah et al., 2015).

Soil presents a complex ecosystem and potentially influences the survival and functionality of the inoculated bacteria. The heterogeneous environment accommodates various microorganisms, fungi, microscopic or macroscopic soil animals (Fan et al., 2018; Nielsen, Wall and Six, 2015). Effective establishment of plant-bacteria interaction is usually uncertain when in the field application due to competition between the inoculated bacteria and the indigenous soil microbial community and heavy metal toxicity. Contrastingly, the persistence of the inoculated bacteria in the soil may cause environmental concerns. The bacteria disturb the soil health and shift the composition of the microbial community (Płociniczak et al., 2020; Ambrosini, Souza and Passaglia, 2016). It is crucial to examine the effects of introducing foreign bacteria on the soil microbial content, hence providing more insights into the field application (Beans, 2017).

Hyperaccumulator describes the heavy metal-resistant plants with the ability to actively uptake and accumulates 100 to 1000 fold higher heavy metals in shoots than non-hyperaccumulator plants (Muszyńska and Hanus-Fajerska, 2015). One best-known example of arsenic hyperaccumulator, *Pteris vittata* L. (Chinese brake fern) accumulates a large amount of arsenic (between 1442-7526 ppm) from the

contaminated soil (Ma et al., 2001). A strong ROS metabolism and an enhanced transport mechanism contributed to the arsenic resistance of the *Pteris vittata* L. (Yan et al., 2019). Plant detoxification of arsenic of non-hyperaccumulator is usually performed through the complexation of the absorbed As(III) with phytochelatins (PCs), followed by the complex (As(III)-PCs) sequestration into vacuoles (Singh, Misra and Sharma, 2020; Ahsan et al., 2008).

This study aimed to determine the phytoremediation efficiency of *Melastoma malabathricum* L. (locally known as Senduduk), by associating this local plant with an arsenate-reducing bacterium, *Microbacterium foliorum* strain SZ1. A DNA sequencing technique elucidated the effect of plant-bacterium association on the indigenous soil bacteria. Also, the expressed leaf proteome of uninoculated and inoculated plants, in the presence or absence of a phytotoxic arsenate concentration, were compared.

1.2 Problem Statement

Persistence of arsenic in the soil has gained major environmental concern over the years as its accumulation into the food chain deteriorates the public health (WHO, 2018). Although the proposed chemical and physical remediation methods effectively remediate the arsenic-contaminated soil, the application is costly and causing disturbances on the soil native microflora (Ali et al., 2013). Bacteria-assisted phytoremediation is an integrated and environmental-friendly approach to remediate the arsenic-contaminated soil. *Melastoma malabathricum* L. is a local plant previously reported as a potential arsenic bioaccumulator (Selamat, Abdullah and Idris, 2014), while *Microbacterium foliorum* strain SZ1 is an arsenic-resistant plant growth-promoting bacterium that reduces As(V) to As(III) and able to produce siderophores and indole-3-acetic acid (IAA). Both secretions are essential to promoting plant nutrient uptake and root growth (Bahari, 2016). The effects of *Microbacterium foliorum* strain SZ1 on *Melastoma malabathricum* L. growth and arsenic accumulation were determined in order to understand its phytoremediation capacity.

To date, the impacts of the inoculated bacteria on soil bacterial composition as well as the plant biological functions are not fully clarified. As the microbial community affects the arsenic availability and the survival of the inoculated bacteria in soil, this project filled the research gap by elucidating the total bacterial composition in the arsenic-treated soil inoculated with SZ1. As the data on the plant response to arsenic are limited at the proteome level, it is important to identify the proteins in *Melastoma malabathricum* L. leaves under the abovementioned condition to understand the arsenic-induced physiological changes in plants. This work investigated the possible effects of field application and hence highlighted the factors that affected the effectiveness of the application.

1.3 Research Objectives

The objectives of the research were:

1. To determine the effect of *Microbacterium foliorum* strain SZ1 inoculation on the growth of *Melastoma malabathricum* L. in arsenic-treated soil
2. To quantify the arsenic content in plant tissues and soil, and arsenic uptake capacity of *Melastoma malabathricum* L.
3. To determine the effect of the *Microbacterium foliorum* strain SZ1 inoculation on the soil bacterial composition and soil pH
4. To detect the effects of arsenic and *Microbacterium foliorum* strain SZ1 inoculation on *Melastoma malabathricum* L. leaves protein profile

1.4 Scope of Study

This project encompasses multidisciplinary research fields, including environmental toxicology, soil microbiology and plant physiology. *Microbacterium foliorum* strain SZ1 was previously isolated and validated for the arsenate-reducing ability. Roots length, shoot length, and plant dry weight was recorded to investigate

the growth response of *Melastoma malabathricum* L. that treated with four levels of arsenic concentrations (0, 10, 30, 50 ppm). Analysis of arsenic content in the treated soil and the plant roots and shoots were conducted using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Illumina MiSeq 16S rRNA gene sequencing technique assessed the effect of soil inoculation on the indigenous bacterial content of *Melastoma malabathricum* L. rhizosphere. *Melastoma malabathricum* L. leaf protein was extracted, and the concentration was determined by Bicinchoninic Acid (BCA) assay. Identification of protein profile was performed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique followed by the characterization by Liquid Chromatography-Mass Spectrometry (LC-MS).

1.5 Significance of Study

The bacteria-assisted phytoremediation technique aims to achieve a high decontamination efficiency of the plant by increasing the bioavailability of the heavy metals and improving the plant vigor. This study utilized the abundance of local plant, *Melastoma malabathricum* L. and arsenate-reducing bacterium *Microbacterium foliorum* strain SZ1 to discover its phytoremediation potential. Associating plant growth-promoting bacteria (PGPB) with plants to facilitate the phytoremediation process is challenging, as establishing the synergistic plant-bacteria relationship can be vulnerable in the arsenic-contaminated soil. The presented data indicated the rhizosphere community of the treated soil, which determined the effects on the rhizosphere soil inoculated. Next, the profiled leaf proteome of *Melastoma malabathricum* L. revealed the governing proteins of the plant in arsenic-bioaccumulation and detoxification. The combined information of soil microbiology and plant proteomic elucidated the conditions of the rhizosphere and plant after the experiment. This study provided a platform to understand the mechanisms of bacteria-assisted phytoremediation, particularly in the rhizosphere bacterial community and plant physiology.

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