

PHYTOBIAL REMEDIATION OF ARSENIC-CONTAMINATED SOIL USING
Melastoma malabathricum L. AND *Microbacterium foliorum*
STRAIN SZ1

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy

Faculty of Science
Universiti Teknologi Malaysia

MAY 2022

DEDICATION

To Allah, The One and Only

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful.

All praise be to Allah, and His blessing upon the completion of this research. I thank God for all the opportunities, trials, and strength that I have been given to finish writing this thesis. I have had so much experience in this process, not only from the academic point of view, but also from the point of view of personality.

First and foremost, I would like to express my special gratitude to my main supervisor, Dr. Fazilah Binti Abd Manan, and to my co-supervisors, Dr. Shafinaz Binti Shahir, and Dr. Norahim Ibrahim, who have given me the golden opportunity to do this wonderful thesis, which has also helped me to do a lot of research, and I have come to know so many new things that I thank them for. I am also grateful for their guidance, understanding, patience and, most importantly, to complete this thesis, they have provided positive encouragement and a warm spirit. It was a great pleasure and honour to have them as my supervisors.

Secondly, I would also like to thank Dr. Umar Alka and Hajiya Samina Alka, my parents, for their financial and moral support, love, and enthusiastic encouragement, without whom I would never have had so many opportunities. May Allah reward you with the highest rank in Jannatul Firdaus Amin. Special thanks go out to Dr. Umar Bala, my steadfast husband, for being a wonderful mentor to me, and thank you for supporting me. I also thank you for your perseverance and diligence in guiding me through my studies. I would like to thank my parents-in-law, Alh Bala Launi, and Hajiya Fatima Abdullahi, for their love and encouragement. I really appreciated it, and may Allah SWA reward you with Jannatul Firdaus Amin.

Thank you very much to my lab mates Mohamed Redzuan bin Abbas and Song Yu Qing, who have always been there to assist me despite having a heavy workload and their own tasks to investigate. I also want to take this opportunity to thank all the staff of the Department of Biosciences (T02), the Faculty of Science, for their cooperation and assistance. I would also like to say a big thank you to Dr Murtala Liman, Dr Aliyu Chikaji and Family, Dr Maryam Ibrahim, and Family (Iman and Ihsan), Dr Sulaiman Muhammad, Dr Jibrin Ndejiko and Dr Oyewusi Habeebat Adekilekun.

Finally, I am very grateful to my family and friends for your love, support, and prayer in my years of study. Thanks to my dear grandma, Hajiya Umma Aishatu and my brothers and sisters for giving me encouragement and prayers throughout my years of study. Well, thank you so much.

May Allah bless the above-mentioned personalities with success and honor in their lives.

ABSTRACT

Arsenic (As) is an increasing threat across the globe, widely known as a threshold carcinogen, and it is reaching harmful values in several areas of the world. In this present study, the effect of *Microbacterium foliorum* strain SZ1, a plant growth promoting bacteria (PGPB) on inorganic As (arsenate) phytoremediation by *Melastoma malabathricum* L. plants were investigated through morphological, histological analysis and proteomic profiling. The experiments included a control treatment (*M. malabathricum* L. plants without As and bacterial inoculation) and two phytoremediation treatments, *M. malabathricum* L. in As contaminated soil inoculated or uninoculated with *M. foliorum* strain SZ1. The morphological parameter and phytoremediation potential induced by *M. foliorum* strain SZ1 on *M. malabathricum* L. were observed for 90 days. Besides, the histological and proteome analysis was conducted using Transmission Electron Microscopy (TEM) and 2-Dimensional gel electrophoresis (2-DE), respectively. The present study showed that plants did not display any noticeable signs of toxicity, but rather the root and shoot length were significantly increased in the presence of *M. foliorum* strain SZ1. In addition, while the Bioconcentration factor (BCF) was increased in the presence of *M. foliorum* strain SZ1 by 0.3 times, the Transfer factor (TF) of As in the *M. malabathricum* L. plants were decreased, whether in the presence or absence of PGPB. The As phytoremediation treatment with *M. foliorum* strain SZ1 also enhanced uptake of As in root (26%) and shoot (22%) than in the other two phytoremediation treatments (As only and control). The findings showed that *M. foliorum* strain SZ1 inoculation reduced As toxicity by substantially reducing the negative stress effects and increasing shoot and root fresh and dry weight. The repair of As induced structural deformities involving disintegration of cell wall and membranes were observed upon *M. foliorum* strain SZ1 inoculation. After PD-Quest analysis, 9 differentially expressed spots subjected to liquid chromatography-tandem mass spectrometry and 9 spots identified with a significant score. The up regulation of tubulin, nucleoside diphosphate kinase and major allergen during As with bacteria exposure confirmed the amelioration of As induced oxidative stress. The abundance of proteins involved in photosynthesis, energy metabolism, defence, signaling, protein biogenesis and nucleoside *M. malabathricum* L. were found higher in As with bacteria than in As alone exposure. Overall, the present study demonstrates the effectiveness of phytoremediation by *M. foliorum* strain SZ1 on responsive pathways including morphology, histology and proteomic of *M. malabathricum* L. plant.

ABSTRAK

Arsenik (As) merupakan ancaman yang semakin meningkat di seluruh dunia, dikenali secara meluas sebagai karsinogen ambang, dan ia mencapai nilai berbahaya di beberapa kawasan di dunia. Dalam kajian ini, kesan bakteria penggalak pertumbuhan tanaman (PGPB) terhadap fitopemulihan As bukan organik (arsenat) oleh *Melastoma malabathricum* L. disiasat melalui analisis morfologi, histologi dan profil proteomik. Eksperimen tersebut merangkumi rawatan kawalan (tanaman *M. malabathricum* L. tanpa As dan inokulasi bakteria) dan dua rawatan fitopemulihan, (*M. malabathricum* L. dalam tanah yang tercemar As, diinokulasi dengan *M. foliorum* strain SZ1 serta tanpa inokulasi mikrob. Parameter morfologi dan potensi fitopemulihan yang disebabkan oleh *M. foliorum* strain SZ1 pada *M. malabathricum* L. dinilai dalam masa 90 hari. Selain itu, analisis histologi dan proteom dilakukan dengan menggunakan mikroskop elektron transmisi (TEM) dan elektroforesis gel 2-dimensi (2-DE). Kajian ini menunjukkan bahawa tumbuhan tidak menunjukkan tanda-tanda ketoksikan yang ketara, tetapi panjang akar dan batangnya meningkat dengan ketara dengan kehadiran *M. foliorum* strain SZ1. Di samping itu, sementara faktor biokonsentrasi (BCF) meningkat dengan kehadiran *M. foliorum* strain SZ1, faktor pemindah (TF) As pada tanaman *M. malabathricum* L. menurun, sama ada dengan kehadiran atau ketiadaan PGPB. Rawatan fitopemulihan dengan *M. foliorum* strain SZ1 juga meningkatkan pengambilan As dalam akar dan pucuk daripada dua rawatan fitopemulihan yang lain. Pembaikan kecacatan struktur yang disebabkan oleh As yang melibatkan perpecahan dinding sel dan membran diperhatikan semasa inokulasi *M. foliorum* strain SZ1. Kajian ini menunjukkan bahawa inokulasi *M. foliorum* strain SZ1 mengurangkan toksik As dengan cara mengurangkan tekanan negatif secara ketara dan meningkatkan berat kering pucuk dan akar segar. Selepas analisis PD-quest, 9 bintik yang dinyatakan berbeza dikenakan spektrometri massa kromatografi cair dan 9 titik yang dikenal pasti mempunyai skor yang signifikan. Strain yang tergolong dalam spesies *M. foliorum* strain SZ1 terkenal dengan kemampuannya menghasilkan siderofor yang bersinergi dengan akar tanaman dapat mempengaruhi kadar mobilisasi arsenat-ke-arsenit, pengambilan dan translokasi As secara efektif. Peraturan tubulin, nukleosida difosfat kinase dan alergen utama semasa pendedahan bakteria As mengesahkan peningkatan tekanan oksidatif yang disebabkan As. Protein yang terlibat dalam fotosintesis, metabolisme tenaga, pertahanan, isyarat, biogenesis protein dan nukleosida *M. malabathricum* L. didapati lebih tinggi pada bakteria di dalam As daripada pendedahan As sahaja. Secara keseluruhan, hasil kajian ini menunjukkan keberkesanan fitopemulihan oleh *M. foliorum* strain SZ1 terhadap laluan tindakbalas dari segi morfologi, histologi dan protein dalam tumbuhan *M. malabathricum* L.

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LIST OF ABBREVIATIONS

As	-	Arsenic
As (V)	-	Arsenate
As (III)	-	Arsenite
Fe	-	Iron
Zn	-	Zinc
Cu	-	Copper
Cd	-	Cadmium
Cr	-	Chromium
Pb	-	Lead
Hg	-	Mercury
N ₂	-	Nitrogen
Pst	-	Phosphate specific transport
Pit	-	Inorganic phosphate transport
ROS	-	Reactive oxygen species
ATP	-	Adenosine triphosphate
GLpF	-	Aquaglyceroporin
SAM	-	S-adenosyl methionine
GSH	-	Glutathione
TMA	-	Trimethylarsine
TMAO	-	Trimethylarsine Oxide
DNA	-	Deoxyribonucleic acid
ACC	-	aminocyclopropane-1-carboxylic acid
PGPE	-	Plant growth promoting endophytic bacteria
PGPR	-	Plant growth promoting rhizospheric bacteria
ISR	-	Induced disease resistance

LIST OF SYMBOLS

<	-	Smaller than
>	-	Greater than
°C	-	Degree Celsius
cm	-	Centimetres
Eh	-	Redox potential
g	-	Gram
gL ⁻¹		Gram per litre
H ⁺		Hydrogen ion
M		Molar
ml		Millilitre
Mm		Millimetre
Ppm		Part per million
v/v		Volume per volume

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CHAPTER 1

INTRODUCTION

1.1 Problem Background

Arsenic (As) contamination of the environment is fast becoming a global problem originating from anthropogenic activities such as urbanization, metal mining, rapid industrialization, and other agrochemicals (Chauhan *et al.*, 2020). Mining activities and various heavy industries are important economic sources for Malaysia, with most of the urban regions and industries found in the western part of the country (Ahmadpour *et al.*, 2012; Rajoo *et al.*, 2013). The common origin of As contamination in Malaysian soils are pesticides, manufacturing industries, abandoned mining sites and inappropriate waste recycling. For example, Tasik Biru, formed from an open gold mining pit, contained a high level of As in the water (Bahari *et al.*, 2017a). Also, a report in a local newspaper revealed a high concentration of As in fishes caught in Sungai Pengorak traced to contamination by waste from bauxite mining in Pahang (Shah, 2015).

International Agency for Research on Cancer and the US Environmental Protection Agency classified arsenic as one of the most dangerous pollutants that is toxic to plants, animals, and human beings. The introduction of this toxic metalloid into the food chain poses long-term risks to human health (Mandal and Suzuki, 2002). Long-term exposure to As can cause genetic variation that can lead to cancers of the skin, lung, and kidney (Flora *et al.*, 2020). About 70 countries and over 150 million people worldwide living in rural areas have been exposed to As through groundwater supplies for many decades (Shankar and Shanker, 2014). Having understood the global problem associated with As contamination and its effect on the global economy and civilization, its concentration from soil and water becomes a primary ecological necessity and concern.

Phytobial remediation implies the combination of microorganisms and plants to reduce the level of heavy metals in soil. The bacteria play an essential role in the ecosystem; they create stable microbial communities that derive significant biogeochemical processes, assist growth in plants and assist in taking up toxic material (Van Der Heijden *et al.*, 2008). These mutual associations can help plants to overcome As-toxicity through development of an antioxidant resistance system and the compartmentalization of thiolated arsenic conjugates (decreasing the amount of free-As) with the help of glutathione (GSH) (Santoyo *et al.*, 2016). These mechanisms help to avoid and overcome damages triggered by As-instigated oxidative stress (Meharg and Hartley-Whitaker, 2002). This process initiates differentiated regulation of proteins, for example, enzymes that catalyze GSH metabolism and ascorbate-glutathione (ASC-GSH) cycle become activated (Ahsan *et al.*, 2009; Requejo and Tena, 2006).

Melastoma malabathricum L. (*M. malabathricum*) is a type of hyper accumulative plant found in the south eastern part of Asian countries (Abdullah and Yong, 2007). They are habitually seen in the wooded area, lowland, and tropical acid soil. This plant can accumulate Al, As, Zn, Pb (Ashraf, 2013; Chua and Hashim, 2008; Yeo and Tan, 2011). It is called Pokok Senduduk in Malay, and it is generally used as a medicinal plant. Besides, it is a hyperaccumulator that tolerates heavy metals contaminants and yields enormous quantities of biomass (Selamat *et al.*, 2014).

The combination of plants and microbes can improve the efficiency of phytoremediation. *Microbacterium foliorum* strain SZ1 (*M. foliorum*) is a genus of mycobacterium from family *Microbacteriaceae*. *Microbacterium* has 96 species. They are gram-positive (Corretto *et al.*, 2015a), essential in agriculture, (Behrendt *et al.*, 2001) and contribute a lot to the soil system. For example, they help the plant to fix nitrogen in the soil in exchange for plant saccharide (Behrendt *et al.*, 2001). *M. foliorum* strain SZ1 is As resistance bacteria isolated in Malaysia from the ex-gold mining site, highly contaminated with As. These bacteria can tolerate As toxicity at a half-maximal inhibitory concentration (IC50) of 140 mM (Bahari *et al.*, 2016).

Dark purple *M. malabathricum* L. was selected for this study as it was reported by different authors to have the capacity in accumulating heavy metals in substantial amount. The use of microbe-assisted bacteria and *M. malabathricum* for As removal and its effect on the physiological, biological and metabolic response of the plant are still at an early stage. Therefore, more studies are needed on the capacity of *M. malabathricum* to take up As when inoculated with bacteria. The aim of the study was to determine the effect of As on plant growth, morphology, uptake, phytoremediation potential and differential protein abundance when the plant is inoculated with the plant growth promoting bacterium.

1.2 Problem Statement

Many environmental studies have focused on the remediation of arsenic contamination in drinking water rather than soil. The high existence of arsenic contaminants in the soil highlights the necessity to search for more remediation strategies. In this context, phytobial remediation is not only an eco-friendly alternative to the conventional bioremediation strategies but also a viable and sustainable approach for the removal of arsenic pollutants from the soil. In the past, *M. malabathricum* L. was used to bioremediate other pollutants in the soil (Patek-Mohd *et al.*, 2018; Mahmud and Burslem, 2020). Also, the phytotoxicity of varied levels of arsenic morphological, histological and proteomic changes, with *M. foliorum* strain SZ1 on the *M. malabathricum* L. species has not been researched, nor their roles in supporting the *M. malabathricum* L. phytoremediation of arsenic-polluted soils. *M. foliorum* strain SZ1 was selected for this study because it has been shown to grow in diverse environments, including soil with adverse conditions, plants, water, and humans (Corretto *et al.*, 2015b), while *M. malabathricum* L. is a plant that can accumulate different metals such as aluminium, lead, and copper (Selamat *et al.*, 2014; Watanabe *et al.*, 1998). The present study contributes to the growing knowledge of the phytobial remediation of contaminated soils through exploration of the potential of the *M. malabathricum* L. species as well as the possibilities of biologically assisted phytoremediation.

1.3 Research Objectives

The objectives of the research are:

- (a) To determine the effects of *M. foliorum* strain SZ1 inoculation on plant growth and root histological characteristics of *M. malabathricum*.
- (b) To determine the changes in As content in soil, leachate and plant tissues after inoculation of *M. foliorum* strain SZ1 on *M. malabathricum*.
- (c) To determine the phytoremediation potential of *M. malabathricum*.
- (d) To examine the changes in differential protein abundance in *M. malabathricum* inoculated with *M. foliorum* strain SZ1 using 2D-PAGE.

1.4 Significance of the Research

This study aims to develop a novel and sustainable bioremediation strategies that remediate contaminated soil using a combination of plants and plant growth promoting bacteria (PGPB). It also explores phytobial remediation as a more effective and sustainable method that allows assimilation of facts on the role played by arsenate-reducing bacteria in As transformation. The findings of this study will enable a better understanding of the use of phytobial remediation in enhancing morphology/ plant growth under As contamination that leads to changes in As speciation, root morphology and protein contents in plant tissue because of *M. foliorum* strain SZ1 inoculation. This research is of great benefit to Malaysia and other countries on their efforts to reduce As contamination, and this will be improved significantly to meet the current standard of Arsenic level worldwide. This study is the first to report on the use of *M. foliorum* strain SZ1 inoculation on *Melastoma malabathricum* L. to reduce a certain level of As contamination.

1.5 Scope of the Research

The present research provides insight into As treatment using phytobial (plant and bacteria) approach. Arsenate was selected because it is the most common arsenic species in soil (Ma *et al.*, 2001). *M. malabathricum* able to survive in arsenic-contaminated soil up to 2,800 ppm (Selamat *et al.*, 2014). The three months old plants were inoculated with *M. foliorum* strain SZ1 that was previously isolated from a gold ex-mining site in Johor. There were four treatments of As in the respective amended soil: 0, 50 and 70 ppm. Each treatment was replicated four times, with a total of 88 pots. The attesting As reducing microbes were inoculated on the plant-soil to compare their treatment efficiency. The containers were arranged in a Randomized Complete Block Design (RCBD) in a greenhouse. Pot experiments were conducted by inoculating the *M. foliorum* strain SZ1. Plant harvested after three months of treatment, and morphological, histological observation and proteome profiling were determined.

The morphological properties of plants were investigated by measuring/observing plant growth/ length, root length, leaf length, shoot length, fresh and dry weight of root and shoot. In addition, histological observations using Transmission Electron Microscopy (TEM) was conducted with the focus on root tissue as it is the closest part of the plants that directly interact with the soil community. Previous study reported the ability of *Melastoma malabathricum* L. roots to take up more As than other parts of the plants. The plant and soil As content were determined using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent Technologies). The phytoremediation potential was determined by phytoremediation capacity by studying arsenic content in tissues/ leachate/ soil to get the uptake level using the Bioconcentration factor (BCF) and Transfer Factor (TF). Changes in differentially displayed proteins in roots were studied by using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE), followed by liquid chromatography- mass spectrometry (LC/MSMS).

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LIST OF PUBLICATIONS

1. Alka, S., Shahir, S., Ibrahim, N., Chai, T. T., Bahari, Z. M., & Abd Manan, F. (2020). The role of plant growth promoting bacteria on arsenic removal: a review of existing perspectives. *Environmental Technology & Innovation*, 17, 100602. **[Q1- Elsevier]**.
2. Alka, S., Shahir, S., Ibrahim, N., Ndejiko, M. J., Vo, D. V. N., & Abd Manan, F. (2021). Arsenic removal technologies and future trends: A mini review. *Journal of Cleaner Production*, 278, 123805. **[Q1- Elsevier]**.
3. Alka, S., Shahir, S., Ibrahim, N., Rahmad, N., Haliba, N., & Abd Manan, F. (2021). Histological and proteome analyses of *Microbacterium foliorum*-mediated decrease in arsenic toxicity in *Melastoma malabathricum*. *3 Biotech*, 11(7), 1-17. **[Q3-Springer]**.
4. Assessment of plant growth promotion properties and impact of *Microbacterium foliorum* for arsenic removal in *Melastoma malabathricum* (*Under review*) **[Submitted to Bioremediation journal–Q4]**