# USING SILICATE SOLUBILIZING BACTERIA AS BIOCONTROL AGENT TO SUPPRESS *Rigidoporus microporus* CAUSING WHITE ROOT ROT DISEASE OF RUBBER

By

NURUL SHAKIRAH BINTI AYOB

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**December 2021**

**Chair : Mohd Yusof Bin Abd Samad, PhD**
**Faculty : Agriculture**

White root rot disease (WRD) caused by Rigidoporus microporus is the most prevalent disease in rubber plantation. Current treatment using chemicals has caused environmental pollution and health hazard. An alternative curative for WRD using microbes to degrade naturally occurring silica (Si) in soil has potential to reduce disease incidence of WRD. Si is a beneficial element for plant growth and its amount in soil is abundant. However, Si availability is relatively low due to strong bonding with other elements in soil. The most important aspect in this research is to make full use of the role of microbes to mineralize silicon in soil for plant uptake against stresses despite promoting plant growth. Thus, the goals of this research were: (i) to isolate and screen potential microbes for its ability in mineralizing silicon, suppressing fungal growth and secreting plant growth promoting (PGP) traits, (ii) to study mechanisms of silicon solubilization by bacteria and (iii) to evaluate efficacy of silicate solubilizing bacteria (SSB) applied at different rates on determination of silicon uptake, inhibition of *R. microporus*, stimulation of induced systemic resistance (ISR) and enhancement of plant growth. To reveal the ability of the potential microbes on reducing disease occurrence of WRD, soil samples under rubber rhizosphere of various ages were collected to select bacteria with silicate solubilizing property, antagonism and plant growth promotion traits. Mechanisms underlying silicate solubilization by selected isolates were conducted. Finally, rubber seedling infected with *R. microporus* was challenged with SSB applied at different rates for 16 weeks to evaluate its effectiveness on reducing disease incidence, solubilizing natural silica in soil and promoting plant growth. Results showed that 3 isolates identified by 16S rRNA as *Bacillus* sp. strain NSAMYKJ16 (SSB16), *Proteus* sp. strain NSAMYKJ18 (SSB18) and *Bacillus* sp. strain NSAMYKJ21 (SSB21) were able to mineralize insoluble silicate, inhibit growth of *R. microporus* and produce PGP traits. It was found that all SSB were able to dissociate silicate minerals up to 14 days with reduction in pH of the cultured medium. Tartaric and succinic acids were major acids involved in solubilization process of silicate minerals by all SSB. In the study of the effect of pH, it was found that all SSB released maximum silicic acid at pH 9 tested on quartz. Meanwhile, magnesium trisilicate was best dissociates only by SSB18 observed at pH 3, pH 6 and pH 9. Based on the ability of SSB to suppress *R. microporus*, results showed that treatment SSB18 applied at 25 mL/seedling (D25) had the lowest disease incidence compared to 50 mL/seedling (D50) and 75 mL/seedling (D75). Meanwhile, SSB16 and SSB21 showed the lowest disease incidence at 50 mL/seedling (D50) and 75 mL/seedling (D75) respectively. Total silica accumulation in plant treated with SSB16, SSB18 and SSB21 were the lowest at 50 mL/seedling (D50), 25 mL/seedling (D25) and 75 mL/seedling (D75) respectively. A correlation analysis between disease suppression and silicon content in root indicated positive association suggesting that disease occurrence increased linearly with silicon accumulation. Lignin content in rubber seedling treated with SSB16, SSB18 and SSB21 were higher in treatment D50 (2.88 mg LTGA $g^{-1}$), D25 (2.82 mg LTGA $g^{-1}$) and D75 (3.74 mg LTGA $g^{-1}$) respectively. Similar trend on the increase of peroxidase enzyme was also shown by SSB16, SSB18 and SSB21 at D50 (4.25 unit $mL^{-1}$), D25 (10.75 unit $mL^{-1}$) and D75 (6.88 unit $mL^{-1}$). Association between both ISR compounds with silicon content in root resulted in negative correlation. Similarly, reduction of disease was also strongly associated with the negative correlation with ISR compounds. Strong positive relationship between lignin and peroxidase reflected that stimulation of peroxidase due to the effect of silicic acid in plant affect lignin formation. Application of SSB significantly increased ($P \leq 0.05$) plant growth attributes and fresh plant biomass were significantly affected by the rate with the highest shown by D50 treatment. Application of SSB increased plant nutrient availability particularly potassium with D50 and D75 rates. Hence, results of this study confirmed that organic acids and pH plays important role in silica dissociation and application of SSB had successfully reduced WRD incidence in rubber seedling.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENGGUNAAN BAKTERIA PELARUT SILIKA SEBAGAI AGEN KAWALAN BIO UNTUK MERENCAT *Rigidoporus microporus* PENYEBAB PENYAKIT AKAR PUTIH TANAMAN GETAH**

Oleh

**NURUL SHAKIRAH BINTI AYOB**

**Disember 2021**

**Pengerusi : Mohd Yusoff Bin Abd Samad, PhD**
**Fakulti : Pertanian**

Penyakit akar putih (WRD) disebabkan oleh kulat *Rigidoporus microporus* merupakan penyakit yang berleluasa dalam perladangan getah. Rawatan terkini menggunakan kimia menyebabkan pencemaran alam sekitar dan merisikokan kesihatan. Rawatan alternative bagi WRD menggunakan mikrob untuk melarutkan silika (Si) semulajadi di dalam tanah berupaya mengurangkan insiden penyakit WRD. Si merupakan unsur bermanfaat bagi tumbesaran tanaman dan jumlahnya di dalam tanah adalah tinggi. Namun, kedapatan Si di dalam tanah adalah rendah kerana terikat oleh unsur lain di dalam tanah. Aspek yang paling utama di dalam kajian ini adalah untuk mengeksplorasi fungsi mikro bagi melarutkan silika di dalam tanah untuk pengambilan tanaman melawan tekanan selain meningkatkan tumbesaran tanaman. Justeru, objektif kajian ini adalah: (i) untuk memencil dan menyaring mikrob yang berupaya melarutkan silika, merencat pertumbuhan kulat dan menghasilkan ciri-ciri penggalak pertumbuhan tanaman, (ii) untuk mengkaji mekanisma pelarutan silika oleh bakteria dan (iii) untuk menilai keberkesanan bakteria pelarut silika (SSB) yang diuji pada kadar berlainan bagi penentuan pengambilan silika, perencatan *R. microporus*, perangsangan aruhan rintangan sistemik (ISR) dan peningkatan pertumbuhan tanaman. Bagi membuktikan keupayaan mikrob mengurangkan kejadian penyakit WRD, sampel tanah di kawasan akar pokok getah daripada pelbagai usia dikumpulkan bagi memilih bakteria yang mempunyai ciri pelarut silika, anatgonis, dan ciri-ciri perangsang pertumbuhan tanaman. Mekanisma disebalik pelarutan silika oleh bakteria kemudiannya dijalankan. Akhir sekali, anak pokok getah dijangkitkan dengan kulat *R. microporus,* dan dirawat dengan SSB pada kadar berbeza selama 16 minggu bagi menguji keberkesanannya mengurangkan penyakit, melarutkan silika semulajadi di dalam tanah dan merangsang tumbesaran pokok. Keputusan menunjukkan bahawa 3 pencilan yang dikenalpasti menggunakan 16S rRNA sebagai *Bacillus* sp. strain NSAMYKJ16 (SSB16),

*Proteus* sp. strain NSAMYKJ18 (SSB18) dan *Bacillus* sp. strain NSAMYKJ21 (SSB21) berupaya melarutka silika, merencat pertumbuhan *R. microporus* dan menghasilkan ciri-ciri perangsang pertumbuhan tanaman. Didapati kesemua strain berupaya melarutkan silika sehingga 14 hari dengan penurunan pH di dalam media kultur. Asid tartaric dan suksinik merupakan asid utama terlibat dalam proses pelarutan silika oleh kesemua SSB. Dalam kajian kesan pH ke atas pelarutan silika, didapati kesemua SSB menghasilkan asid silisik maksima pada pH 9 yang diuji ke atas kuartza. Sementara itu, pada magnesium trisilika, pelarutan yang paling baik adalah pada pH 3, pH 6 dan pH 9 yang kesemuanya ditunjukkan oleh SSB18. Berdasarkan keupayaan SSB untuk merencat *R. microporus,* keputusan menunjukkan rawatan SSB pada kadar 25 mL/anak pokok (D25) mempunyai insiden penyakit yang terendah berbanding 50 mL/anak pokok (D50) dan 75 mL/anak pokok (D75). Sementara itu, SSB16 dan SSB21 menunjukkan insiden penyakit yang terendah pada kadar rawatan masing-masing pada 50 mL/anak pokok (D50) dan 75 mL/anak pokok (D75). Jumlah silika terkumpul di dalam tanaman yang dirawat dengan SSB16, SSB18 dan SSB21 adalah terendah pada kadar rawatan masing-masing 50 mL/ anak pokok (D50), 25 mL/ anak pokok (D25) dan 75 mL/ anak pokok. Analisa korelasi antara rencatan penyakit dan jumlah silika di dalam akar menunjukkan hubungan yang positif yang mencadangkan bahawa kejadian penyakit meningkat secara selari dengan jumlah silika terkumpul. Kandungan lignindi dalam anak pokok getah dirawat dengan SSB16, SSB18 dan SSB21 masing-masing adalah tinggi pada kadar D50 (2.82 mg LTGA $g^{-1}$), (2.88 mg LTGA $g^{-1}$) dan D75 (3.74 mg LTGA $g^{-1}$). Trend yang sama pada peningkatan enzim peroksida juga ditunjukkan oleh SSB16, SSB18 dan SSB21 pada kadar D50 (4.25 unit $mL^{-1}$), D25 (10.75 unit $mL^{-1}$) dan D75 (6.88 unit $mL^{-1}$).Hubungan antara kedua-kedua kompaun ISR dengan kandungan silika di dalam akar menunjukkan korelasi negatif. Trend serupa juga ditunjukkan oleh pengurangan penyakit dengan korelasi negatif antara pengurangan penyakit dan kompaun ISR. Hubungan positif yang kuat antara lignin dan enzim peroksida menunjukkan rangsangan enzim peroksida hasil daripada asid silisik memberi kesan kepada pembentukan lignin. Penggunaan SSB yang meningkatkan sifat-sifat pertumbuhan tanaman dan jisim segar dengan ketara ($p \leq 0.05$) dipengaruhi oleh kadar aplikasi dengan bacaan tertinggi ditujukkan oleh rawatan D50. Penggunaan SSB meningkatkan kedapatan nutrient tanaman khususnya potassium yang ditunjukkan pada kadar D50 dan D75. Justeru, keputusan kajian menyimpulkan dan mengesahkan asid organik dan pH berperanan penting dalam proses pelarutan silika dan penggunaan SSB telah berjaya mengurangkan kejadian penyakit WRD pada anak pokok getah.

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**Mohd Yusoff Bin Abd Samad, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

**Zulkefly Bin Sulaiman, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

**Siti Izera Binti Ismail, PhD**
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

______________________________
**ZALILAH MOHD SHARIFF, PhD**
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 April 2023

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Signature: ______________________________

Chairman of Supervisory Committee: Mohd Yusoff Abd Samad, PhD

Signature: ______________________________

Member of Supervisory Committee: Zulkefly Bin Sulaiman, PhD

Signature: ______________________________

Member of Supervisory Committee: Siti Izera Binti Ismail, PhD

# TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| µg | Microgram |
| µg/mL | Microgram per millilitre |
| µL | Microliter |
| µM | Micromolar |
| µM | Micrometer |
| $^{0}$ | Degree |
| $^{0}$C | Degree celcius |
| A.R. | Analytical reagent |
| Al | Aluminium |
| Al-O | Aluminium oxide bond |
| AMP | Antimicrobial peptide |
| ANOVA | Analysis of variance |
| AUDPC | Area under disease progression curve |
| C | Carbon |
| C/N | Carbon to nitrogen ratio |
| $C_{10}H_{11}NO_5S$ | l-amino-2-naphthol-4-sulfonic acid |
| $C_8H_{18}O$ | Octyl alcohol |
| Ca | Calcium |
| CEC | Cation exchange capacity |
| cfu | Colony forming unit |
| cm | Centimetre |
| $cmol_c$ | Centimole charge |
| DAI | Day after incubation |

| | |
|---|---|
| DI | Disease incidence |
| DR | Disease reduction |
| DS | Disease severity |
| DSI | Disease severity index |
| ED | Effective dose |
| Fe | Iron |
| FeO | Iron (II) oxide |
| g | Gram |
| *g* | G-force |
| $H^+$ | Hydrogen ion |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulphuric acid |
| $H_4SiO_4$ | Silicic acid |
| ha. | Hectare |
| HCl | Hydrochloric acid |
| HMWOAs | High molecular weight acids |
| $HNO_3$ | Nitric acid |
| IAA | Indole-3-acetic acid |
| ISR | Induced systemic resistance |
| K | Potassium |
| kbp | Kilo-base pair |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| L | Litre |

| | |
|---|---|
| LMWOAs | Low molecular weight acids |
| ln | Natural log |
| LTGA | Lignothioglycolic acid |
| m | Metre |
| M | Molar |
| Mg | Magnesium |
| Mg | Milligram |
| mL | Millilitre |
| Mm | Millimetre |
| mM | Millimolar |
| Mmol | Millimol |
| N | Nitrogen |
| Na | Sodium |
| NaOH | Sodium hydroxide |
| $NH_4F$ | Ammonium fluoride |
| $NH_4OAc$ | Ammonium acetate |
| nm | Nanometre |
| O.D. | Optical density |
| OH | Hydroxide |
| P | Phosphorus |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PDA | Potato dextrose agar |
| PIRG | Percentage inhibition rate growth |

| | |
|---|---|
| pmol | Picomole |
| POX | Peroxidase |
| PROC GLM | Procedure generalized linear model |
| psu | Percent siderophore unit |
| rpm | Revolution per minute |
| rRNA | ribosomal RNA |
| SEM | Scanning electron microscope |
| Si | Silicon |
| SI | Solubilization index |
| Si-O bond | Silicon oxide bond |
| $SiO_2$ | Silicon dioxide |
| SOC | Soil organic carbon |
| SSB | Silicate solubilizing bacteria |
| UV | Ultra violet |
| VOC | Volatile organic compound |
| w/v | Weight per volume |
| WRD | White root disease |
| Zn | Zinc |

# CHAPTER 1

# INTRODUCTION

Rubber (*Hevea brasiliensis*) is one of the major plantation crop and main commodity export in Malaysia after oil palm. The cultivation of rubber had seen a progressed from year to year in the area planted with rubber after it was commercially established in 1904 (Setiawan et al., 2007) which was 2,400 hectares in the early 1900s to 218,900 hectares in 1910s. Later, rubber cultivation grown rapidly and became one of the key success in Malaysian economy which contributes to the gross domestic product (GDP) and national gross export up to 50% as well as becoming source of income for small holders. However, the area grown with rubber was reduced due to several constraints such as emergence of synthetic rubber and disturbance of its growth by plethora of diseases. Biotic stress affecting growth of rubber due to diseases had been a major concerned to researchers at global.

Like other crops, various pathogenic diseases influence rubber production (Faÿ et al., 2010). According to Wastie (1975), rubber is a plantation crop that subjected to a plethora of economically important pathological diseases. White root disease (WRD) caused by basidiomycetes fungus *Rigidoporus microporus* is one of the challenging disease posing an epidemic treat in rubber plantation. It is well known destructive disease that infect rubber growing countries in Cameroon, Nigeria, India, Thailand, Indonesia, Ivory Coast, Ghana, Gabon, Sri Lanka, Malaysia and West and Central Africa (Wastie, 1975; Wattanasilakorn et al., 2015). Guyot & Flori (2002) reported that the disease has caused extensive death of rubber and severe loss that lead to the reduction of the tree stand and yield of latex being produced.

In order to maintain the steady supply of natural rubber, preventing and controlling the occurrence of WRD is necessary to prevent the widespread of the disease to the neighbouring trees. Currently, numerous strategies such as breeding of resistant clone, management of cultural practices and application of chemical fungicides had been employed to control the disease. According to (Narayanan & Mydin K., 2012), the commercially cultivated rubber clones has a very narrow genetic base due to its originality produce from hybridization thus there are no known species of rubber clone reported to be resistant towards *R. microporus.* Despite breeding, proper clearing of the land by uprooting the soil and poisoning the old rubber tree before replanting had been known to reduce the disease occurrence but this method requires high cost for small holders. The emergence of chemical fungicide was known to be effective against white root disease but the drawback of chemical usage on environment and health hazard has gained awareness to find for an alternative treatment.

Application of microbes as in biological control in agricultural practice has been considered as a viable alternative to chemical fungicide that can conserve and sustain the environment (Ouda, 2014) which suppress the activities of one organism by one or more other organism. In case of biological control for white root disease of rubber, previous studies showed that *Trichoderma harzianum, Trichoderma hamatum* (Jayasuriya & Thennakoon, 2007), *Aspergilus niger, Chaetomium bostrychodes, Chaetomium cupreus* (Kaewchai & Soytong, 2010), *Hypocrea virens, Hypocrea jecorina, Hypocrea lixii, Trichoderma spirale* (Nicholas et al., 2015), and *Streptomyces* sp. (Nakaew et al., 2015) had significant effect on the reduction of the severity index of disease. Although biological control agents have the ability to suppress the disease, but the efficacy of the introduced biocontrol agent in the field must be fully understood since there are many interrelationships between the antagonist, pathogen, the host plant and other indigenous organisms in the soil. Therefore, selection of the antagonist should be stressed initially starting from isolation to screening of potential antagonist so that the selected biocontrol agent does not only reduce the severity of plant disease but also promote plant growth.

Soil plays a vital role in plant health and fertility as it provides nutrients and protect plant from pathogen. This undoubtedly is related to the complex nature of soil microbial communities in soil ecosystem. In natural soil ecosystem, the diversity and number of microbial communities is higher because of high biomass content compared to the agro-ecosystem soil which had been polluted and had low amount of organic matter. As a result, plant becomes weakening when it is infected with pathogen. Therefore, management practices are essential to reclaim soil microorganisms. Different microorganisms carried out different function and show different performance according to its specified host plant. Variety of microorganisms are available for biological control but not all microorganisms have the ability to produce antagonism, plant growth promoting properties and solubilize naturally occurring mineral in soil.

Currently, research on microorganism capable of solubilizing silicate mineral in soil and supressing phytopathogenic fungi had gained attention by researchers worldwide. Silicate solubilizing bacteria like *Bacillus flexus, Bacillus mucilaginosus, Bacillus megaterium and Pseudomonas fluorescens* were known to have antagonistic, plant growth promoting properties and solubilized insoluble silica (Vasanthi et al., 2018). Silicon which is the second most abundant mineral in soil was found to be effective in controlling various pests and diseases caused by both fungi and bacteria in different plant species (Ma, 2004). However, most of the research conducted on dissolution and uptake of silica were tested on monocotyledonous plant like rice, wheat, maize and sugarcane. On the other hand, it is important to study the uptake of silica on dicotyledonous plant especially the perennial crop of woody stem. Nonetheless, silicon research had gained attention only in the last two decades (Nawaz et al., 2019). Though silicon was abundant in soil but it is not an indication that it is supplied sufficiently because soluble silicon in the form of monosilicic acid ($H_4SiO_4$) in soil system is regulated by soil pH, and amount of clay, organic matter, minerals and Fe / Al oxides/hydroxides which are associated with the geologic age of the soil (Tubana & Heckman, 2015). In other words, the degree of soil weathering determined the amount of silica in the soil. In Malaysia, most of the soil used for agricultural activity is highly weathered soil which are from the order Oxisol and Ultisol (Jusop & Ishak, Weathered tropical soils: the ultisols & oxisols, 2010). About 70% of Malaysian soil are from these two types of soil order which are mainly used for rubber and oil palm cultivation. Oxisol and Ultisol contained high amount of complex Al/Fe hydroxide due to the disintegration of silicate mineral as a result of intense weathering. Quartz is the most common mineral found in Malaysian weathered soil and the most stable mineral in the soil. Because of its physico-chemical properties and stable form, availability of soluble silicon in soil solution is less between 1.6 – 1.9 mg $L^{-1}$ of dissolved silicon (Dress et al.,1985). With respect to low solubility, dissolved silicon are easily removed from soil solution due to the adsorption by Fe and Al hydroxide (Bruun et al., 1994). Previous studies reported that Fe and Al hydroxide can remove large amount of dissolved silicon because they have strong adsorption capacity. Therefore, introduction of microorganism like bacteria and fungi are essentially important in the dissolution process of insoluble silica in soil. Studies showed that some bacteria and fungi had the ability to mineralize silica and silicates in nature by several modes of action such as ligands of cation, secretion of organic and inorganic acids, alkali process by production of ammonia and amine and production of extracellular polysaccharides (EPS) by the bacteria at acidic pH (Konhauser, 2015). Microbes with the ability to mineralize insoluble silica for plant uptake in strengthening plant cell wall from pathogen attack is potentially to have double protection for plant against pathogen. Thus, this study was conducted with the following objectives:

1. To isolate and screen potential microbes for its ability in mineralizing silicon, suppressing fungal growth and secreting plant growth promoting characteristics.

2. To study mechanisms of silicon solubilization by potential isolates.

3. To evaluate the efficacy of silicate solubilizing bacteria applied at different rates on the silicon uptake, inhibition of *R. microporus,* stimulation of induced systemic resistance and enhancement of plant growth under rain shelter house.

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