RHIZOSPHERIC MICROORGANISMS FROM *Hevea brasiliensis* AND THEIR ANTAGONISM ON *Rigidoporus microporus*

Aizat Shamin Noran
Production Development Division
Malaysian Rubber Board, 47000 Sungai Buloh,
Selangor, Malaysia
aizat@lgm.gov.my

Nor Afiqah Maiden
Production Development Division
Malaysian Rubber Board, 47000 Sungai Buloh,
Selangor, Malaysia
afiqah@lgm.gov.my

Mohd Adi Faiz Ahmad Fauzi,
Production Development Division
Malaysian Rubber Board, 47000 Sungai Buloh,
Selangor, Malaysia
adifaiz@lgm.gov.my

ABSTRACT

The rhizosphere is one of the most complex environments that are influenced by plant roots and active microhabitat where plant roots and microbes interact. Composition of the rhizosphere microbiota can negatively or positively influence plant characteristics such as stress tolerance, health, development, and productivity. The present study aimed to select rhizospheric microorganisms which are antagonistic to Rigidoporus microporus, the causal agent of white root disease of Hevea brasiliensis. 53 rhizospheric microorganisms comprises of 38 bacteria and 15 fungi were successfully isolated from five different rubber growing areas in Malaysia and were tested in vitro against R. microporus using dual culture inhibition assay technique. 28 microorganisms comprises of 16 bacteria and 12 fungi showed an antagonistic activity against R. microporus with a range of inhibition between 20.57% and 87.52%. Dual culture inhibition assay is a simple and efficient technique for quick screening of antagonistic microorganisms. However, the limitation of using dual culture inhibition assay was the production of antimicrobial substances was only restricted around microbial colonies on the plate and no direct contact between antimicrobial substances with the pathogen. Dipped stick method provide direct interaction between antagonist and fungal pathogens. 19 antagonistic microorganisms were selected and undergo secondary screening process using dipped stick method. 17 antagonistic microorganisms showed an inhibition of R. microporus growth with a range 14.17 and 96.33%. The results from this study provide an encouraging basis for the utilization of biological control agent for an effective control of white root disease of Hevea brasiliensis.

Key words: *Hevea brasiliensis*, white root disease, *Rigidoporus microporus*, rhizospheric microorganisms, biocontrol agent

Introduction

Among the root disease of *Hevea brasiliensis* (Willd. Ex Adr. & Juss) Mull. Arg., white root disease is the most destructive in the most of the rubber growing countries. Root diseases are an important economic factor to rubber industry since it kills rubber trees irrespective of age. White root disease caused more tree losses compare to either red root disease or brown root disease especially from the first to the fourth year after planting (Ng and Yap, 1990). The most important method for controlling white root disease is to isolate and destroy the food base or infected dead wood. Control of root diseases required fulfillment of three basic principles; land preparation before planting, effective detection rounds and eradication of all infected trees. If these three basic principles are followed accordingly, the incidence of the disease in replanted areas can be reduced to below economic threshold level.

Chemical treatment procedure is a quite effective if the diseased trees are detected at an early stage. Therefore, the most economic and effective way to control white root disease is to identify the infection at a very early stage and treat accordingly. Chemical treatment method using collar protectant dressing is fairly cheap and is resistant to decomposition in the soil (RRIM, 1964). However, it is quite tedious and labour extensive. The technique of fungicide drenching is an attractive technique as it involves little labour compare to application of collar protectant. Tilt (Propiconazole), Bayleton (triadimefon), Bayfidan (triadimenol), Folicur (tebuconazole) and Contaf (hexaconazole) are effective systemic fungicide to control white root disease caused by *R. oligoporus* (Hashim and Chew, 1997; Jayaratne et al., 2001).

The indiscriminate use of fungicides may lead to accumulation of toxic residues, development of fungicide resistance, and environmental pollution (Jayasuriya and Thenakoon, 2007). In this context, the utilization of biocontrol approaches may help to develop an eco-friendly control strategy for managing plant diseases. Biological control using microorganism is among options

apart from fungicides in controlling plant diseases. Many species of actinomycetes, bacteria and fungi have the ability to serve as potential antagonists and to be an effective control agent of plant disease. Antagonists express a variety of mechanisms to inhibit the pathogen including the induction of plant resistance, competition for nutrient or space, antifungal metabolites production, biosurfactant production and mycoparasitism (Zhang and Pierson, 2001). *Pseudomonas fluorescens* employ a wide range of antagonistic mechanism such as production of inhibitory compounds, siderophores production, and nutrient or site competition (Bull et al., 1991).

Most microorganisms used in biological control were already present in the natural environment. The general techniques for the development of antagonistic bacteria involve isolation of a large number of microorganisms from different site. Primary selection of potentially useful microbes can be done in the laboratory (in-vitro), and followed by field testing (in-vivo) under normal ecology of the pathogen and also the antagonists. The real potential of antagonistic microorganisms as a biological control depends upon its establishment and ability to maintain its threshold population under field condition. Many factors affected the successful application of biocontrol agents including temperature, moisture, pH, and availability of oxygen. The quantity and quality of available nutrients are major determinants of microbial population size and the metabolite activity

The flow steps on development of biopesticides include screening an identification of antagonists, mass production, survival of antagonist in extreme conditions, formulation, application techniques, toxicology, registration and commercialization. Formulations affect many aspects in the development of successful biopesticides including improve the shelf life of the product, enhance the ability of the antagonistic microorganism to proliferate and survive in extreme environment, improve effectiveness for disease control and enhance product stability during storage. Formulation has shown to improve the antagonists shelf life and performances under field condition. Biopesticides for controlling phytopathogenic fungi are formulated in variety of ways such as wettable powders, dusts, gels, emulsions, pellets, and granules. The talcum-based formulations have been used for the management of several crop diseases in India (Bharathi et al., 2004). Mixing of chitin with antagonistic bacteria in the formulation has been found to increase the antagonists population in the culture medium and increase biocontrol efficacy (Bharathi et al., 2004).

The general objective of the study is to develop microorganism-based biopesticide for the control of *R. microporus*, the causal agent of white root disease in rubber and the specific objective of this study were to isolate and identify antagonistic microorganisms against *R. microporus*.

Research design

Isolation of Antagonistic Microorganisms

Isolation of antagonistic microorganisms were conducted by serial dilution and plating method using nutrient agar and Rose-bengal agar for bacteria and fungi, respectively (Chang and Yang, 2009). Each colonies growth on the plates will be purified onto a fresh medium to obtain a pure culture.

Screening of Antagonistic Microorganisms against *R. microporus* using Dual Culture Inhibition Assay

Each pure culture of antagonistic microorganisms were screened against *R. microporus* for antagonistic activity using dual culture assay**.** 5 mm diametric mycelial plug of 6-day old culture of *R. microporus* will be placed at the centre of a potato dextrose agar (PDA) plate. The potential antagonistic microorganism will be streaked on PDA medium with a distance of 2.5 cm between *R. microporus* agar plug and the potential antagonistic microorganism**.** Percent inhibition radial growth (PIRG) between *R. microporus* and the potential antagonistic microorganisms were observed and calculated using the following formula:

$$\text{PIRG (\%)} = (1 - (\text{fungal growth/control growth})) \times 100\%$$

Screening of Antagonistic Microorganisms against *R. microporus* using Dipped Stick Method

Preliminary study to observe the interaction between selected antagonistic microorganisms with *R. microporus* on the rubber wood was conducted using dip stick method. Antagonistic bacteria were grown in nutrient agar (NA) for 2 days. Cell culture suspension of antagonistic bacteria were collected by scraping 2-days culture of antagonistic bacteria Antagonistic fungi were grown in potato dextrose agar (PDA) for 7-days. Spores and mycelium of antagonistic fungi were collected by scraping 7-days culture of antagonistic microorganisms grown on PDA using sterilized distilled water. Rubber wood (10 cm long and 1.5 cm in diameter) were soaked in spores and mycelium suspension of antagonistic microorganisms for 2 minutes. Then, the rubber wood was transferred into test tube containing *R. microporus* cultures. The test tubes were incubated for 10-days when *R. microporus* in control tubes has fully covered rubber woods. Percent inhibition of *R. microporus* growth were observed and calculated using the following formula:

$$\text{Percent Inhibition (\%)} = (1 - (\textit{R. microporus} \text{ growth(treatment)} / \textit{R. microporus} \text{ growth growth(control)})) \times 100\%$$

Results

Isolation of Antagonistic Microorganisms and Screening of Antagonistic Microorganisms using Dual Culture Inhibition Assay

A total of 53 microorganisms were successfully isolated from Sungai Buloh Experimental Station (SPSB), Kota Tinggi Experimental Station (SPKT), Sungai Sari Experimental Station (SPSS), Hutan Simpan Rantau Panjang and Taman Bukit Tawau. The number of fungal and bacterial from each location were presented in Table 1.

Table 1: Fungal and bacterial isolates obtained from different locations in Malaysia

Locality	Number of fungal isolates	Number of bacterial isolates	Total
SPSB, Selangor	3	12	15
Hutan Simpan Rantau Panjang, Batu Arang	4	3	7
SPSS, Kedah	2	11	13
SPKT, Johor	2	7	9
Taman Bukit Tawau	4	5	9
Total	15	38	53

15 microorganisms were isolated from SPSB, 7 microorganisms were isolated from Hutan Simpan Rantau Panjang, 13 microorganisms were isolated from SPSS, 9 microorganisms were isolated from Taman Bukit Tawau and 9 microorganisms were isolated from SPKT. Dual culture inhibition assay were conducted on 53 isolated microorganisms and percent inhibition for each of the isolates were calculated. Data was collected on the 10th day of incubation when the *R. microporus* in the control set has completely covered the plate. Results of dual culture inhibition assay for microorganisms isolated from SPSB, Hutan Simpan Rantau Panjang, SPSS, Taman Bukit Tawau and SPKT are shown in Table 2.

Table 2: Percent Inhibition of Radial Growth (PIRG) of *R. microporus* by antagonistic microorganisms

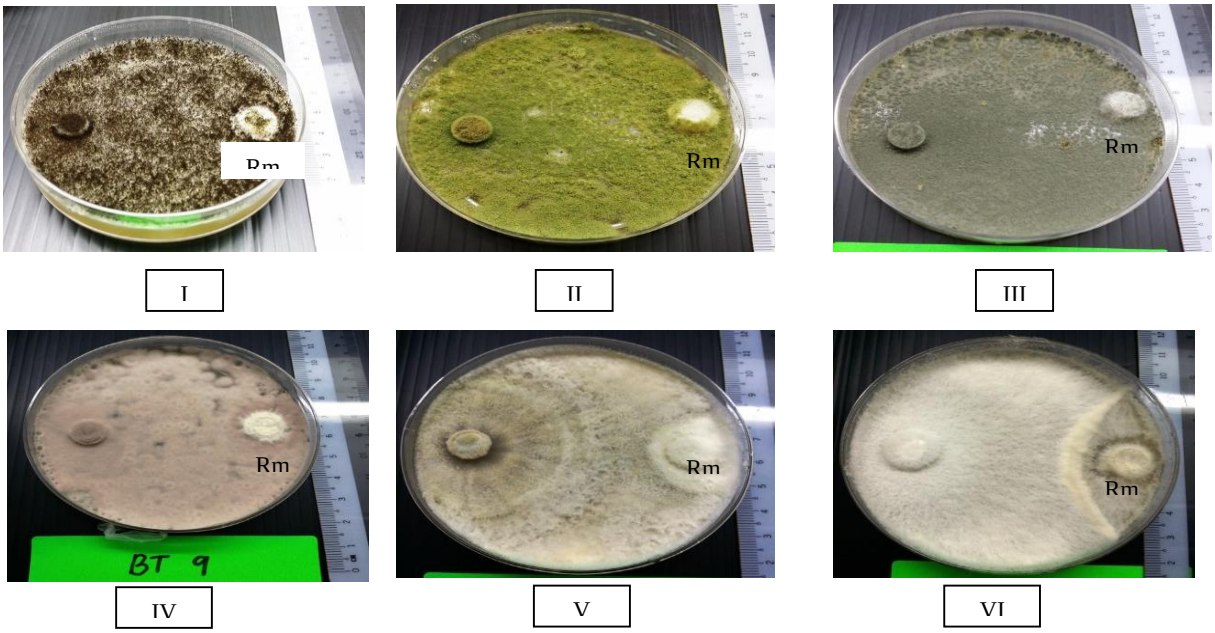
Microorganisms	**Percent Inhibition of R. microporus growth (%)**
SB15 – F4/3 (Fungi)	87.53 ± 0.56^{a}
SB1 – SB F1 (Fungi)	87.45 ± 1.01^{a}
SB10 – F 5/1 (Fungi)	87.22 ± 0.68^{a}
BT9 (Fungi)	$85.10 \pm 1.12^{a, b}$
BT3 (Fungi)	$83.02 \pm 1.58^{b, c}$
KT9 (Fungi)	81.05 ± 1.22^{c}
SB8 – 3 (Bacteria)	55.60 ± 0.95^{d}
BT2 (Fungi)	54.77 ± 2.54^{d}
SB9 – 4 (Bacteria)	50.01 ± 0.80^{e}
SB6 – 1/2 (Bacteria)	$47.80 \pm 1.22^{e,f}$
SB13 – 5/3 (Bacteria)	$46.70 \pm 0.43^{f, g}$
SB2 – AntiR/4/2 (Bacteria)	$44.40 \pm 0.96^{g, h}$
SB14 – 5/5 (Bacteria)	43.50 ± 1.28^{h}
SB5 – 1/4 (Bacteria)	$42.20 \pm 1.10^{h, i}$
KT7 (Bacteria)	$40.56 \pm 1.34^{i, j}$
BT4 (Fungi)	$39.11 \pm 1.7^{j, k}$
SB3 – RL/4 (Bacteria)	$38.90 \pm 0.99^{j, k}$
SS11 (Fungi)	$38.49 \pm 1.26^{j, k}$
SS13 (Bacteria)	37.41 ± 1.93^{k}
BA3 (Bacteria)	$36.60 \pm 1.16^{k, l}$
BA6 (Fungi)	$36.48 \pm 1.03^{k, l}$
BA4 (Fungi)	34.50 ± 1.36^{l}
BA7 (Fungi)	27.56 ± 1.27^{m}
SS4 (Bacteria)	$26.02 \pm 2.2^{m, n}$
SS9 (Bacteria)	$25.76 \pm 1.06^{m, n}$
KT6 (Bacteria)	$23.52 \pm 1.48^{n, o}$

SS1 (Bacteria)	22.96 ± 1.31$^{o, p}$
BA1 (Bacteria)	20.57 ± 0.95^{p}
SB11 – 5/1/2 (Bacteria)	0.00^{q}
SB12 – 5/2 (Bacteria)	0.00^{q}
SB4 – 1/3 (Bacteria)	0.00^{q}
SB7 – 2 (Bacteria)	0.00^{q}
KT1 (Bacteria)	0.00^{q}
KT2 (Bacteria)	0.00^{q}
KT3 (Bacteria)	0.00^{q}
KT5 (Bacteria)	0.00^{q}
KT8 (Bacteria)	0.00^{q}
KT10 (Fungi)	0.00^{q}
BT1 (Bacteria)	0.00^{q}
BT5 (Bacteria)	0.00^{q}
BT6 (Bacteria)	0.00^{q}
BT7 (Bacteria)	0.00^{q}
BT8 (Bacteria)	0.00^{q}
SS2 (Bacteria)	0.00^{q}
SS3 (Bacteria)	0.00^{q}
SS5 (Bacteria)	0.00^{q}
SS6 (Bacteria)	0.00^{q}
SS7 (Bacteria)	0.00^{q}
SS8 (Bacteria)	0.00^{q}
SS10 (Bacteria)	0.00^{q}
SS12 (Fungi)	0.00^{q}
BA2 (Bacteria)	0.00^{q}
BA5 (Fungi)	0.00^{q}
Control	0.00^{q}

Values are expressed as mean ± standard error of six replications. Mean followed by the same letter are not significantly different ($P<0.05$) as determined by Duncan multiple range test

Out of 53 microorganisms isolated from five different locations, only 28 microorganisms comprises of 16 bacteria and 12 fungi showed an antagonistic activity against *R. microporus* with range of inhibition between 20.57% and 87.52%. There were six microorganisms showed a good antagonistic activity against *R. microporus* with PIRG more than 80% with code F4/3 (SB15), SBF1 (SB1), F5/1 (SB10), BT9, BT3, and KT9 with the percentage of inhibition 87.53, 87.45, 87.22, 85.10, 83.02, and 81.05%, respectively.

Figure 1: F4/3 (I), F5/1 (II), SBF1 (III), BT9 (IV), BT3 (V) and KT9 (VI) showed a good antagonistic activity against *R. microporus* with PIRG more than 80%



Screening of Antagonistic Microorganisms using Dipped Stick Method

18 microorganisms were selected for secondary screening using dipped stick method namely SBF1, F5/1, F4/3, BT3, BT2, BT9, KT9, BT4, SS9, KT6, SB8, SB6, SB9, KT7, SB13, SB2, BA3, and BA1. Results for screening of antagonistic microorganisms using Dipped Stick Method were shown in Table 3.

Table 3**:** Percent Inhibition (%) of *R. microporus* by antagonistic microorganisms using dipped stick method

Microorganisms	**Percent Inhibition of R. microporus growth (%)**
Tilt (20mL/L)	100.00[a]
Tilt (10mL/L)	100.00[a]
BT3 (Fungi)	96.33 ± 0.80[b]
F 5/1 (Fungi)	91.50 ± 0.43[c]
SB8 (Bacteria)	85.33 ± 0.84[d]
SS9 (Bacteria)	83.83 ± 0.91[d]
SB F1 (Fungi)	77.67 ± 0.61[e]
BA3 (Bacteria)	75.00 ± 1.32[e,f]
KT7 (Bacteria)	74.50 ± 1.96[f]
F4/3 (Fungi)	71.33 ± 0.88[g]
SB13 (Bacteria)	67.67 ± 1.41[h]
BA1 (Bacteria)	50.17 ± 1.19[i]
BT4 (Fungi)	46.67 ± 1.02[j]
KT6 (Bacteria)	44.83 ± 1.35[j]
SB9 (Bacteria)	39.33 + 0.99[k]
BT2 (Fungi)	36.17 ± 1.11[l]
BT9 (Fungi)	29.00 ± 0.86[m]
KT9 (Fungi)	14.17 ± 1.3[n]
SB2 (Bacteria)	0.00[o]
SB6 (Bacteria)	0.00[o]
Control	0.00[o]

Values are expressed as mean ± standard error of six replications. Mean followed by the same letter are not significantly different (P<0.05) as determined by Duncan Multiple Range Test.

Out of 18 microorganisms tested, only 16 microorganisms showed an inhibition of *R. microporus* growth with range of inhibition between 14.17 to 96.33%. 4 microorganisms showed a good antagonistic activity with inhibition of *R. microporus* more than 80% compare to control which are BT3, F5/1, SB8, and SB9 with percent of inhibition 96.33, 91.50, 85.33, and 83.83% respectively. Tilt 250EC (Sygenta) at 10mL/L and 20 mL/L were served as positive control.

Figure 2: Dipped Stick method for screening of antagonistic microorganisms activity against *R. microporus*

Discussion

Antagonistic microorganisms are potential useful source for the control of *R. microporus.* The results in this study showed that several isolated soil microorganisms were able to inhibit the growth of *R. microporus* in *in vitro* study. Microbial pesticides express a variety of mechanisms to inhibit the pathogen including the induction of plant resistance, competition for nutrients or spaces, antifungal metabolites production, cell-wall degradation and lytic enzymes, siderophores production, biosurfactant production and mycoparasitism (Zhang and Pierson, 2001).

Dual culture plate assay is a simple technique for the screening of antagonistic microorganisms. This method was also very efficient and effective for quick screening of antagonistic microorganisms that produced antimicrobial substances. However, the limitation of using dual culture plate assay was the production of antimicrobial substances was only restricted around microbial colonies on the plate and no direct contact between antimicrobial substances with the pathogen. Dipped stick method provide direct interaction between antagonist and fungal pathogens. There are 10 antagonistic microorganisms which showed better inhibition of *R. microporus* by dipped stick method. The antagonistic microorganisms are BT3, F5/1, SB8, SS9, BA3, KT7, SB13, BA1, BT4, and KT6. This shows that these isolates require direct contact with the fungal pathogen to provide a better antagonistic activity against *R. microporus.*

Dipped stick method provide an advantage to *R. microporus* where the food base (rubber wood) was provided. There are 8 antagonistic microganisms which showed a reduction of *R. microporus* growth when tested using dipped stick method. The antagonistic microorganisms are SBF1, F4/3, SB9, BT2, BT9, KT9, SB2, and SB6. Bacteria isolates, SB2 and SB6 inhibit the growth of *R. microporus* with PIRG of 44.40% and 47.80% by using dual culture plate assay. However, there are no inhibitions when both isolates were tested using dipped stick method. This indicated that both, SB2 and SB6 were not able to survive on the rubber wood. This showed that it is required to study on nutrient requirement of potential antagonistic microorganisms to ensure the effectiveness of biocontrol agent under field conditions where nutrient can be a limiting factor.

Conclusion

53 microorganisms were successfully isolated from five different rubber growing areas in Malaysia. Dual culture inhibition assay (*in vitro*) has been conducted using 53 obtained microorganisms and percent inhibition of radial growth (PIRG) for each of the isolates has been calculated. Out of 53 microorganisms isolated, only 28 microorganisms comprises of 16 bacteria and 12 fungi showed an antagonistic activity against *R. microporus* with a range of inhibition between 20.57% to 87.52%. Out of 28

antagonistic microorganisms, six microorganisms showed good antagonistic activity against *R. microporus* with PIRG more than 80%. Secondary screening of 18 antagonistic microorganisms was conducted using dipped stick method. Sixteen out of 19 antagonistic microorganisms showed an inhibition of *R. microporus* growth with a range 14.17 to 96.33%. Since the conditions in soil are much more complex than those *in vitro*, nursery and field trial will be established in the future to investigate the effectiveness of the antagonistic microorganisms to control white root disease under *in vivo* conditions.

References

Bharathi, R., Vivekananthan, R., Harish, S., Ramanathan, A. and Samiyappan, R. (2004). Rhizobacteria-based Bio-formulations for the Management of Fruit Rot Infection in Chillies. *Crop Protection,* 23, 835-843

Bull, C.T., Weller, D.M., and Thomashow, L.S. (1991). Relationship Between Root Colonization and Suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* Strain 2-79. *Phytopathology*, 81, 954-959

Chang, C.H., and Yang, S.S. (2009). Thermo-tolerant Phosphate-solubilizing Microbes for Multi-functional Biofertilizer Preparation. *Bioresource Technology*, 100, 1648-1658

Hashim, I. and Chew, B.H. (1997). Effects of Integrating *Trichoderma* and Fungicides on Control of White Root Disease of *Hevea* Rubber. *Journal of Natural Rubber Research*, 12, 43-57

Jayaratne, R., Wettasinghe, P.C., Siriwardene, D., and Peiris, P. (2001). Systemic Fungicides as a Drench Applications to Control White Root Disease of Rubber. *Journal of Rubber Research Institute of Sri Lanka,* 84, 1-17

Jayasuriya, K.E. and Thennakoon, B.I. (2007). Biological Control of *Rigidoporus microporus*, The Cause of White Root Disease in Rubber. *Ceylon Journal of Science (Biological Science),* 36, 9-16

Ng, K.Y. and Yap, T.H. (1990). The Effects of Triadimefon and Triadimenol in Controlling White Root Disease of Rubber. In *Proceedings of 3rd International Conference on Plant Protection in the Tropics, Malaysia,* (pp 31-35)

Rubber Research Institute of Malaysia (RRIM) (1964). Collar Protectant Dressing Against White Root Disease. *Planter's Bulletin*, 70, 8-10

Zhang, Z. and Pierson, L.S. (2001). A Second Quorum-sensing System Regulates Cell Surface Properties but Not Phenazine Antibiotic Production in *Pseudominas aureofaciens. Applied and Environmental Microbiology*, 67, 4305-4315