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Isolation of rhizospheric and endophytic soil bacteria SPLUMS-1 and SPLUMS-2 of oil palm against *Ganoderma* sp. JN234427

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ABSTRACT

Aims: The present study aimed to identify the potential ERB (endophytic root bacterium) and RSB (rhizospheric soil bacterium) of young oil palm trees against *Ganoderma* sp. JN234427, the causal pathogen of oil palm basal stem rot in Sabah.
Methodology and results: Dual culture test was performed to select indigenous isolates with antagonistic character against *Ganoderma* sp. JN234427. Eleven RSB and fifteen ERB isolates were isolated from 5-year-old oil palm plots in the Faculty of Sustainable Agriculture, Universiti Malaysia Sabah. Gram's test and morphological features of all isolates were recorded and eight bacteria isolates with potential antagonistic activity were identified using the 16S rRNA gene sequences and Gen-III Biolog MicroStation™ automated identification system. The RSB_3 *Pseudomonas* sp. SPLUMS-1 is one of the elite isolates with highest percentage inhibition of radial growth PIRG activity (57.5 %), followed by ERB_{15} *Burkholderia* sp. SPLUMS-2 (48.1 %), RSB_4 *P. tolaasii* (44.3%), and RSB_5 *Bacillus subtilis* (33.4 %). The PIRG activity of *Pseudomonas* sp. SPLUMS-1 and *Burkholderia* sp. SPLUMS-2 were further evaluated using agar amendment, culture filtrate and mycelia growth tests. The PIRG of *Pseudomonas* sp. SPLUMS-1 for the three tests mentioned above were 95.6 %, 100 % and 100 % respectively. Meanwhile, significant differences (p<0.05) in PIRG value were recorded between the agar amendment (20.1 %), culture filtrate (89.0 %) and mycelia growth (67.3 %) tests for *Burkholderia* sp. SPLUMS-2.
Conclusion, significance and impact of study: Three bacterial genera consisting of *Pseudomonas*, *Bacillus* and *Burkholderia* were revealed in this study. The RSB_3 and ERB_{15} isolates are potential biological control agents against *Ganoderma* sp. JN234427 for oil palm plantations in Sabah.

Keywords: Burkholderia, *Pseudomonas*, *Ganoderma* sp. JN234427, endophyte, rhizosphere

INTRODUCTION

Rhizosphere can be ascribed as the zone of soil associated with plant roots, and is generally rich in root exudates, exchangeable nutrients, minerals as well as biological niche. These soils provide an important 'sink' or living habitat for a wide variety of microorganisms, ranging from pathogenic to beneficial bacteria species that at least in part of its reproduction cycles demand plant organic carbon (source). Plant growth promoting rhizobacteria (PGPR) refers to a group of symbiotic bacteria that are capable of enhancing plant growth and nutrients uptake through secretion of growth regulator signaling compounds such as the 3-indole acetic acid (auxin) (Ajay *et al*., 2012) and organic acid that helps in mobilization of soil nutrients such as the insoluble phosphorus from soil colloid (Archana *et al*., 2012). In addition, PGPR might also contribute to plant health by being able to exert several biological control mechanisms through competition for space and nutrients, as well as secretion of lytic enzymes (Suryanto *et al*., 2012) and biological active compounds (Keel *et al*., 1990). These PGPR can be further divided into two main groups: 1. Intracellular PGPR or endophytic bacteria that live within or at least during part of their life cycle inside a plant; and 2. Extracellular PGPR or rhizospheric bacteria (Gray and Smith, 2005).

The oil palm plot at the FSA (Faculty of Sustainable Agriculture), Universiti Malaysia Sabah (UMS) was once an industrial plantation where no basal stem rot (BSR) incidents have ever been reported. This opened up the opportunity to carry out preliminary screenings upon this infection-free plot for potential biological specimens that might be colonized in this natural suppressive soil and capable of protecting palm trees from harmful pathogens especially the BSR fungal pathogen *Ganoderma boninense* (Sapak *et al*., 2008).

MATERIALS AND METHODS

Ganoderma boninense culture

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Pure culture of *Ganoderma* sp. JN234427 (JN234427) from the Sustainable Palm Oil Research (SPOR) unit, UMS was used (Chong *et al*., 2013). The fungus was subcultured and grown for two cycles on PDA in the dark at 30 °C to ensure optimum mycelial growth had been redeemed before screening tests.

Root and soil samplings

Thirty samples of oil palm root and rhizospheric soil were randomly collected from ten selected young 5-year-old oil palm trees at the oil palm plot of FSA, UMS. Soil was sampled using auger to a depth of 15 cm at about 3 ft around the trunk under the oil palm canopy according to standard soil sampling protocol and roots of pencil-thick size exposed from the same sampling site were sampled on the basis of three samples per tree.

Isolation of bacteria

ERB were isolated from the oil palm roots according to Sapak *et al*. (2008). RSB from soil (50 g) were isolated through the preparation of soil suspensions (500 mL) and a series of dilution processes (Ajay *et al*., 2012). Pure culture from a single colony was maintained on nutrient agar for Gram's staining test and morphological characterization. A corresponding bacterial stock was maintained in nutrient broth (NB) stored in a refrigerator at 4 °C for assay and identification purposes.

***In vitro* assays**

Antagonism study of ERB and RSB isolates towards *Ganoderma* sp. JN234427 was done using dual culture assays arranged in completely randomized design with five replicates. PIRG was calculated when the mycelium of the *Ganoderma* sp. JN234427 had reached the edge of the control plates that had been inoculated with sterilized blank NB (Idris *et al*., 2008). PIRG was defined as [(R1-R2/R1) x 100%], where (R2) denotes the radius growth of the *G. boninense* towards the antagonist colony and (R1) denotes the radial growth of *G. boninense* in the control plate. Potential ERB and RSB bacteria with 50% PIRG were further screened using agar amendment and culture filtrate tests (Bivi *et al.*, 2010) as well as mycelia growth test, according to Rahamath *et al*. (2010).

Statistical analysis

The mean difference of PIRG was analyzed using one-way ANOVA and Post hoc LSD test ($p < 0.05$).

Bacteria identification

Bacteria with strong PIRG value were identified using Gen III Biolog MicroStation™ identification system according to the manufacturer's manual using IF-A broth and an incubation period of 24 h at 33 °C. In addition, these bacteria were also identified using 16S rRNA gene sequences approach. DNeasy Blood and Tissue kit (QIAGEN) was used to extract microbial genomic DNA according to the manufacture procedures. The bacterial primer set used to amplify the 16S rRNA region in this study were BSF820 (Forward: 5'-AGA GTT TGA TCC TGG CTC AG-3') and BSR1541 (Reverse: 5'-AAG GAG GTG ATC CAG CCG CA-3'). Amplified PCR products were purify using QIAquick PCR Purification Kit (QIAGEN) before they were sent out for sequencing. Resulted 16S rRNA sequences were analyzed using NCBI's Basic Local Alignment Search Tool (BLAST) for identification purposes.

RESULTS AND DISCUSSIONS

Isolation and identification of ERB and RSB

Fifteen ERB and eleven RSB were isolated from symptomless young oil palm trees. Their morphological characteristics, antagonistic screening results expressed in PIRG, and possibly identification are summarized in Table 1. Based on the bacteria enumerated from nutrient agar, Gram-negative rod shaped bacteria dominated the young plantation soil with the genus pseudomonad being the most abundant. In contrast, only one Gram-positive bacterium *Bacillus subtilis* was isolated from the rhizospheric soil and these findings are in agreement with the results reported by Nur Masirah *et al*. (2011). The only ERB that has showed potential PIRG activity was ERB_{15}, whereas seven RSB isolates that consisted of three bacterial species as identified using the Biolog MicroStation™, have exhibited moderate to strong PIRG activities with RSB_3 showing the highest among others. The three possible homologous from the NCBI GenBank database for ERB_{15} and RSB_3 were identified as shown in Tables 2a and 2b, respectively.

Agar amendment and culture filtrate tests

Based on preliminary PIRG screening results, strains ERB_{15} and RSB_3 were chosen for further evaluation. In the agar amendment test (Figure 1a-c), the mean growth of *Ganoderma* sp. JN234427 mycelia in PDA agar amended with ERB_{15} and RSB_3 were 2.26 cm (PIRG: 20.1 %) and 0.12 cm (PIRG: 95.6 %) respectively in comparison to the control. In culture filtrate test (Figure 1d-f), the mean growth of *Ganoderma* sp. JN234427 mycelia in PDA agar amended with ERB_{15} culture filtrate was 0.31 cm (PIRG: 89.0 %) and no growth was observed in the agar plate amended with RSB_3 culture filtrate (PIRG: 100 %).

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Table 1: Morphological characteristic of root associated bacteria from the young oil palm plantation and their antagonistic activity against *Ganoderma* sp. JN234427.

Bacterial isolate	Colony morphology	Margin	Elevation	Shape	Gram's Stain	PIRG (%) activity	I.D (Genus)
ERB_1	Round	Irregular	Crateriform	Rod	G(–)	+	ND
ERB_2	Round	Smooth	Convex	Rod	G(–)	+	ND
ERB_3	Concentric	Ciliate	Flat	Rod	G(–)	+	ND
ERB_4	Round with scalloped margin	Ciliate	Flat	Rod	G(–)	-	ND
ERB_6	Irregular and spreading	Lobate	Raised	Rod	G(–)	+	ND
ERB_7	Irregular and spreading	Wavy	Flat	Rod	G(–)	-	ND
ERB_9	Round with raised margin	Wavy	Raised	Rod	G(–)	-	ND
ERB_{12}	Rhizoid	Lobate	Raised	Rod	G(–)	+	ND
ERB_{13}	Filamentous	Branching	Raised	Spheric	G(–)	-	ND
ERB_{14}	Round with scalloped margin	Lobate	Raised	Rod	G(–)	-	ND
ERB_{15}	Irregular	Wavy	Umbonate	Rod	G(–)	++ (48.10^{ab})	*Burkholderia* sp. SPLUMS-2
ERB_{16}	Irregular and spreading	Irregular	Raised	Spheric	G(–)		ND
ERB_{17}	Round with scalloped margin	Lobate	Raised	Rod	G(–)	-	ND
ERB_{18}	Irregular and spreading	Irregular	Flat	Rod	G(–)	-	ND
ERB_{20}	Round	Smooth	Convex	Rod	G(–)	-	ND
RSB_1	Irregular	Undulate	Flat	Rod	G(–)	-	ND
RSB_2	Circular	Undulate	Flat	Rod	G(–)	-	ND
RSB_3	Irregular	Undulate	Flat	Rod	G(–)	+++ (59.00^{a})	*Pseudomonas* sp. SPLUMS-1
RSB_4	Irregular	Lobate	Flat	Rod	G(–)		*Pseudomonas tolaasii*
RSB_5	Irregular	Branching	Raised	Rod	G(+)	++	
RSB_6	Irregular	Lobate	Flat	Rod	G(–)	(48.65^{ab})	*Bacillus subtilis*
RSB_7	Circular	Entire	Flat	Rod	G(–)	++ (33.35^{c})	ND
RSB_8	Irregular	Lobate	Flat	Rod	G(–)	-	ND
RSB_9	Irregular	Undulate	Flat	Rod	G(–)	- ++ (39.71^{b})	*Pseudomonas tolaasii*
RSB_{10}	Irregular	Undulate	Flat	Rod	G(–)	+++ (56.44^{a})	*Pseudomonas* sp. SPLUMS-1
RSB_{11}	Irregular	Lobate	Convex	Rod	G(–)	+++ (57.13^{a}) ++ (44.52^{b})	*Pseudomonas* sp. SPLUMS-1 *Pseudomonas tolaasii*

Note: EB, endophytic bacteria; RB, rhizospheric bacteria; G(–), Gram negative; G(+), Gram positive; +++, strong (>50%); ++, moderate (25-50%); +, weak (<25%); -, no activity; ND, not determined; Figure followed by different alphabet showed significant difference at $p<0.05$; ID, Identification of bacteria were made by using Gen III Biolog MicroStation™ and 16S rRNA gene sequence.

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Table 2a: The most homologous three microorganisms from the NCBI gene bank in comparison to the ERB_{15} *Burkholderia* sp. SPLUMS-2.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP003774.1	*Burkholderia cepacia* GG4 chromosome 1, complete sequence	2713	5409	100%	0.0	100%
FJ606689.1	*Burkholderia* sp. 2xiao7 16S ribosomal RNA gene, partial sequence	2713	2713	100%	0.0	100%
AY946011.1	*Burkholderia cepacia* strain RRE5 16S ribosomal RNA gene, partial sequence	2713	2713	100%	0.0	100%

Table 2b: The most homologous three microorganisms from the NCBI gene bank in comparison to the RSB_3 *Pseudomonas* sp. SPLUMS-1.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HE978271.1	*Pseudomonas aeruginosa* partial 16S rRNA gene, type strain DSM 50071T	2719	2719	100%	0.0	100%
JX035794.1	*Pseudomonas aeruginosa* strain N002 16S ribosomal RNA gene, partial sequence	2719	2719	100%	0.0	100%
JQ894531.1	*Pseudomonas* sp. CEBP1 16S ribosomal RNA gene, partial sequence	2719	2719	100%	0.0	100%

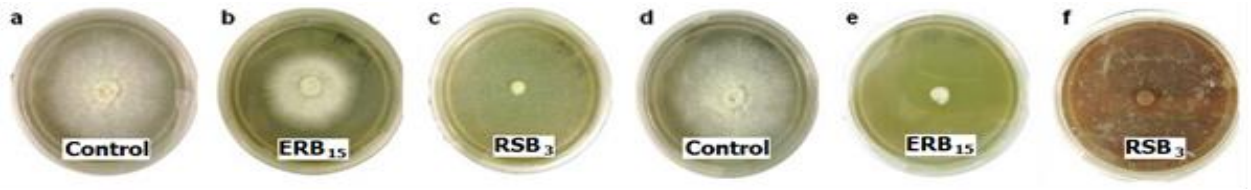

Figure 1: Effect of root associated bacterial isolate ERB_{15} *Burkholderia* sp. SPLUMS-2 and RSB_3 *Pseudomonas* sp. SPLUMS-1 on the radial growth of *Ganoderma* sp. in bacterial amended test (a-c) and culture filtrate test (d-f).

Mycelia growth test

RSB_3 exhibited strong antifungal activity with nearly no visible growth (PIRG: 100 %) observed from the mycelia plug that had been previously dipped into the bacterial suspension. Microscopic examination of the RSB_3 treated mycelia plug revealed swelling and bursting of hyphae that led to malformation of the mycelial mass as compared to the normal hyphal structures in the control. It is suggested that this fungicidal effect of RSB_3 towards *Ganoderma* sp. JN234427 involves the secretion of antibiotics (Keel *et al*., 1990) although no direct evidence was obtained in this study. Meanwhile, ERB_{15} also demonstrated significant inhibitory activity towards *Ganoderma* sp. JN234427 (PIRG: 67.3 %). Microscopic examination revealed low density of hyphal mat compared to the control. No clear hyphae abnormality was observed but the fungistatic effect of this bacterium had significantly suppressed the mycelia growth of *Ganoderma* sp. JN234427 in comparison to the control plate (Figure 2). The potential use of this bacterium on *Ganoderma* sp. also has been reported by Kamaruzaman and Dikin (2005) and Azadeh *et al*. (2010).

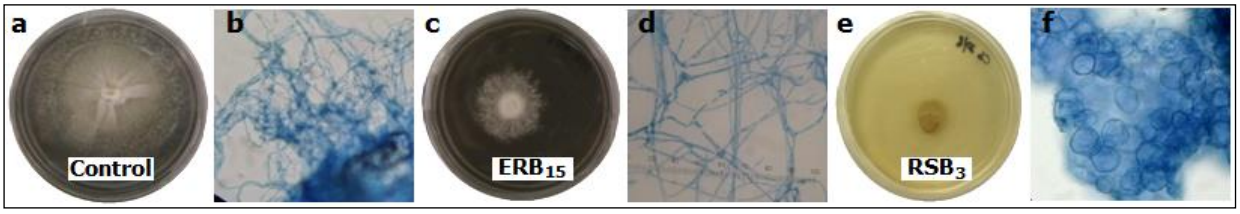

Figure 2: Mycelia growth and microscopic examination on hyphae of *Ganoderma* sp. JN234427 dipped in sterile nutrient broth (control) (a-b), ERB_{15} *Burkholderia* sp. SPLUMS-2 (c-d) and RSB_3 *Pseudomonas* sp. SPLUMS-1 (e-f) suspensions (Magnification = 400×)

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