Malaysian Journal of Microbiology, Vol 14(7) 2018, pp. 663-673
DOI: http://dx.doi.org/10.21161/mjm.108617

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Diversity and functional characterization of antifungal-producing *Streptomyces*-like microbes isolated from the rhizosphere of cajuput plants (*Melaleuca leucodendron* L.)

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Received 10 August 2017; Received in revised form 7 August 2018; Accepted 8 August 2018

ABSTRACT

Aims: The study was undertaken to evaluate the diversity of actinomycetes from the rhizosphere of the cajuput plant (*Melaleuca leucodendron* L.) using ARDRA, and to examine their *in vitro* antifungal potency against selected fungi.
Methodology and results: A total of 78 *Streptomyces*-like microbes were isolated from the limestone rhizosphere of cajuput plants and cultured in SN agar medium. The ability to inhibit fungal growth was observed using a dual culture assay. The diversity of the isolates was examined by morphological and genotype profiling using ARDRA (Amplified 16S ribosomal DNA restriction analysis), following which they were assigned to eight groups. Seventeen (21.8%) strains showed the ability to produce the antifungal compound as evidenced by their antagonism *in vitro* against the tested fungi, namely *Saccharomyces cerevisiae* BY 47420, *Candida albicans* CGMCC 2538, *Aspergillus flavus* NRLL 3357, and *Fusarium oxysporum* KFCC 11363P in the dual culture assay. One isolate, GMR22, which showed potent antagonism against unicellular and filamentous fungi, displayed 97% 16S rRNA sequence similarity to *Streptomyces vayuensis*.
Conclusion, significance and impact of study: This study demonstrated that a diversity of *Streptomyces*-like antifungal producing microbes were present in the rhizosphere of cajuput plants in the Wanagama Forests of Indonesia. This rhizosphere represents a potential new source of actinomycetes that produce biologically active compounds. One selected strain (GMR22) has the potential to be developed as a commercial biofungicide.

Keywords: Antifungal, *Streptomyces*-like microbes, *Melaleuca leucadendron* L., limestone soil, rhizosphere

INTRODUCTION

Actinomycetes have been explored extensively for the production of various antibiotics. In recent years, these microbes have been known to inhabit the plant rhizosphere where they colonize plant roots. Actinomycetes have received attention for their ability to stimulate plant growth and to exert biocontrol over a range of phytopathogens (Franco-Correa *et al*., 2010; Wang *et al*., 2013).

The rhizosphere is an environment of plant roots and the surrounding soil, characterised by high microbial activity. Plant roots secrete exudates (approximately 5-21% of photosynthetically fixed carbon) that include organic compounds such as amino acids, fatty acids, organic acids, phenolics, plant growth regulators, plant sterols, and vitamins (Khamna *et al*., 2009). The availability of carbon, nitrogen, and nutrients secreted from plant roots is beneficial to the microbial population, both pathogens and non-pathogens (Lynch, 1983). The composition and quantity of root exudates are highly dependent on the plant species and on abiotic conditions such as moisture content and temperature (Söderberg and Bååth, 1998).

Actinomycetes which are frequently encountered in the plant rhizosphere have become the target of research owing to their ability to inhibit the growth of plant pathogenic fungi (Suzuki *et al*., 1999). They play major roles, both qualitatively and quantitatively, in the rhizosphere by influencing the growth of plants and

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(7) 2018, pp. 663-673
DOI: http://dx.doi.org/10.21161/mjm.108617

protecting them against invasion by plant root pathogens through the production of antibiotics and antifungal agents (Atalan *et al*., 2000; Doumbou *et al*., 2002; Costa *et al*., 2013; Zhu *et al*., 2014). These microbes inhibit the growth of pathogenic fungi by releasing antifungal compounds or enzymes that damage the cell walls of the invading pathogen (Getha and Vikinesway, 2005). Actinomycetes influence plant growth and protect plants through the production of substances such as auxins, antibacteria, and antifungals (Costa *et al*., 2013; Zhu *et al*., 2014).

Many species of actinomycetes, particularly the genus *Streptomyces*, are widely exploited because of their ability to control the growth of pathogenic fungi. Numerous researchers have successfully screened actinomycetes for the production of antifungals from the rhizosphere of such plants, including *Vitis vinifera* L. (Loqman *et al*., 2009), *Argania spinosa* L. (Barakate *et al*., 2002), sagebrush (*Artemisia tridentata*) (Basil *et al*., 2004) and *Zingiber officinale* (Taechowisan and Lumyong, 2003), and cajuput (Rosruen and Pornpakaku, 2008). Nevertheless, there is still a paucity of knowledge on antifungal-producing actinomycetes of the rhizosphere.

Hence, this study aims to screen the actinomycetes from the cajuput plant rhizosphere to evaluate their diversity based on ARDRA, and to examine their *in vitro* antifungal potency.

MATERIALS AND METHODS

Collection of rhizosphere soil sample

Rhizosphere soil samples were collected from Wanagama Forest-Yogyakarta, Indonesia. The samples were randomly collected from where cajuput plants (*Melaleuca leucadendron* L.) were growing, at locations spaced 300 to 500 m apart. The soil samples obtained from a depth of 5 to 15 cm surrounding the roots were placed in sterile 50 mL conical tubes, and stored at 4 °C until isolation of the microbes as described by Lee and Hwang (2002) and Aghighi *et al*. (2005).

Isolation of *Streptomyces*

Ten grams of each soil sample were transferred into a 250 mL Erlenmeyer flask containing 90 mL of sterile distilled water. The suspension was shaken at 200 rpm for 30 min on a rotary shaker before heating in a waterbath at 70 °C for 1 h. The mixture was allowed to settle for 15 min and aliquots of 1 mL supernatant from the soil mixtures were transferred into 9 mL of sterile distilled water, and subsequently to a final dilution of 10^{-6}. Approximately 0.1 mL of the final dilution was spread on plates of Starch Nitrate agar (SNA) containing 20 g soluble starch, 0.5 g NaCl, 1 g KNO_3, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 20 g agar, 1000 mL aquadest (pH 7.2-7.4), and supplemented with cycloheximide at 50 ppm. The plates were incubated at 30 °C for up to 20 days. Pure cultures of *Streptomyces*-like strains were preserved on SN agar slant at 4 °C for further investigation.

Amplified ribosomal DNA restriction analysis (ARDRA)

Extraction of genomic DNA of the strain was conducted as described by Badji *et al*. (2006). An approximately 1500 bp fragment of the 16S rRNA gene was PCR amplified using the following primers: 27f [5'-AGAGTTTGATCCTGG CTCAG-3'] and 1492r [5'-GGTTACCTTGTTACGACTT-3']. The conditions for the amplification of gene fragment were as follows: pre-denaturation of the target DNA at 98 °C for 3 min followed by 30 cycles at 94 °C for 1 min, primer annealing at 57 °C for 1 min, and primer extension at 72 °C for 5 min; the reaction mixture was held at 72 °C for 5 min. The amplification product was separated by electrophoresis on 1.5% (w/v) agarose gel in 0.5× TBE buffer and stained with ethidium bromide (Petrosyan *et al*., 2003; Sacido and Genilloud, 2004). The PCR product of the 16S rRNA gene was purified using Kit Gel Purified (Promega Corporation, USA). Amplicons representing various strains were subjected to ADRDA. Following examination of the ADRDA profile, 10 µL of the PCR product was digested with the restriction enzyme *Hae*III (Promega Corporation, USA) at 37 °C for 3 h. The digested DNA sample was analysed by electrophoresis on 2% agarose gel. ARDRA restriction fingerprints were compared visually and scored manually for the presence or absence of a fragment. The binary data were processed by the MVSP software version 3.1a. The similarity matrix derived using the binary data of the ARDRA restriction fragment was subjected to cluster analysis using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithms.

Evaluation of antifungal-producing strains

The ability to inhibit the growth of fungi was evaluated using the dual culture assay. Agar blocks of *Streptomyces*-like strains (diameter 6 mm) cultured on SNA for 7 days were cut and placed on plates containing fungi (Φ = 30 mm) in the fungal inoculum test (Lee and Hwang, 2002). The tested fungi were *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus flavus*, and *Fusarium oxysporum*. The plate was incubated for 7 days at 28 °C, and the degree of inhibition of fungal mycelial growth was measured from the dual test plate. Each treatment was performed in triplicate. The plate without *Streptomyces* inoculum was used as the control. The inhibition zone was determined as the radius of fungal growth in the direction of a *Streptomyces* colony. Percentage inhibition was graded as >70% (+++, strong inhibition), 50-70 % (++, moderate inhibition), and <50% mm (+/-, weak inhibition) using the following equation:

$$\gamma I = \frac{C - T}{C} x100$$

where, γI=mycelial growth inhibition in percentage,
C=mycelial growth diameter in untreated of *Streptomyces*,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(7) 2018, pp. 663-673
DOI: http://dx.doi.org/10.21161/mjm.108617

T=mycelial growth diameter in treatment of *Streptomyces*.

In vitro antifungal and antibacterial bioassay of selected strains

The selected strain was spot-inoculated onto SNA medium and incubated at 30 °C for 7 days. After this, the antagonistic reaction between the strain and the test microbes (yeast and bacteria) was examined using the agar block assay. All the plates were incubated at 30 °C with an incubation time of 48 h for bacteria and 72 h for yeast. The experiments were carried out in triplicate.

TLC-Bioautography of antifungal from selected strains

The selected strain was grown on production medium (SN broth) for 10 days at 30 °C. Biomass and the supernatant were separated by centrifugation at 3500 rpm for 15 min. The supernatant was extracted with various solvents such as n-hexane, benzene, chloroform, and ethylacetate (1:1 v/v). The extract was concentrated using rotary evaporator and dried with anhydrous Na_2SO_4 before assaying for antifungal activity. The active concentrate was evaluated by Thin Layer Chromatography (TLC)-Bioautography performed using 0.2 mm-thick silica gel plates (Silica gel 60, F_{254}, Merck) as described by Frandberg *et al*. (2000). Ten microliters of concentrate were applied onto the plates, and developed with [$CHCl_3$:MeOH, 20:1(v/v)] as the mobile phase. The TLC plate was placed on agar bearing a culture of the tested fungus. The plates were kept in a refrigerator for 2 h prior to incubation at 35 °C for 2 days. After incubation, the clear zones (active spots) on the culture were noted and their Rf values recorded.

Morphological and physiological characterization of selected strains

Further characterization of the selected strains was performed according to traditional morphological criteria, including colony characterization on plates, spore morphology, hydrolysis of starch, and pigment production, as described by Goodfellow and Cross (1984). The selected strain was grown on Starch Casein agar using the slide culture method; spore morphology was then assessed by light microscopy and scanning electron microscopy (SEM). Utilization of carbohydrates and nitrogen as the sole carbon and nitrogen sources was tested with the medium (ISP9) supplemented with different carbohydrates and nitrogen as sole carbon or nitrogen sources respectively, as described by Shirling and Gottlieb (1966). Growth was observed under different salt concentrations (0-15% w/v at intervals of 0.5%). The temperature range for growth was determined on ISP2 broth using a temperature gradient incubator (4 °C-50 °C).

Analysis of cell wall composition

The determination of diaminopimelic acid (DAP) stereoisomer in whole-cell hydrolysis was performed as a chemotaxonomic marker of actinomycetes. Analysis of DAP of selected strains was conducted using TLC, as described by Nishimura *et al.* (2002).

Sequences of 16S rRNA gene of selected strains

Extraction of genomic DNA from the selected strains was conducted as described above. The amplicon of DNA fragments was sequenced using the ABI 3100 sequencer according to the manufacturer's instructions (ABI PRISMA 3100 Genetic Analyzer User's Manual). Sequences obtained were aligned with those in the GeneBank using the Blast program to determine the closest known relatives based on gene homology. Identities of selected strains were confirmed using analysis of partial 16S rDNA sequences.

RESULTS AND DISCUSSION

Isolation and screening of the selected strain

Seventy-eight *Streptomyces*-like strains were successfully grown on SNA from 7 sampling sites of the *Melaleuca leucadendron* L. limestone soil rhizosphere. The strains were selected based on differences in morphological and culture characteristics. Colonies from the same sampling site that showed the same characteristics were regarded as similar strains to avoid duplication. A total of 17 strains produced antifungal activity based on the dual culture assay, representing about 21.8% of the total number of strains identified (Table 1). While the Wanagama Forest floor was characterised by a rhizosphere dominated by carbonate, it also contained phosphates and other minerals. This condition supported high soil pH and was a habitat favorable to microbes like actinomycetes that thrive in an alkaline environment. The microbes isolated in this study were high in their diversity, and approximately 70% of them were *Streptomyces*. When such microbes colonize plant roots, they play an important role in supplying various nutrients as well as plant growth promotion and biocontrol agents to the plant (Kloepper and Schroth, 1981; Walker *et al*., 2003). Past studies have drawn attention to the distribution and diversity of actinomycetes in rhizosphere habitats (Suzuki *et al*., 2000; Pandey and Palni, 2007; Khamna *et al*., 2009). In a study, a total of 131 actinomycetes screened from the rhizosphere were generally *Streptomyces*-like in character (Bouizgarne *et al*., 2006). Xu *et al*. (1996) reported that among a population of actinomycetes examined in China, about 83% comprised *Streptomyces.* Numerous studies have shown that actinomycetes bacterial communities are more commonly encountered around plant roots than in soil outside of the rhizosphere, showing that microbial activity is better supported in the vicinity of the roots (Crawford *et al*., 1993; Garland, 1996; Sembiring, 2009).

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Table 1: Occurrence of *Streptomyces*-like strains isolated from different sampling sites of limestone soil rhizospheres of the cajuput plant.

Site Sampling	No. of *Streptomyces*-like strains	No. of antifungal producing-*Streptomyces*-like strains	Strain codes
WGF01	11	1	#GMR4
WGF02	6	2	#GMR5, #GMR6
WGF03	10	3	#GMR9, #GMR14, #GMR16
WGF04	15	1	#GMR17
WGF05	11	5	#GMR18, #GMR19, #GMR20, #GMR22, #GMR27
WGF06	12	1	#GMR31
WGF07	22	4	#GMR36, #GMR37, #GMR41, #GMR44
Total	78 (100%)*	17 (21.8%)*	18

*Values in the brackets indicate the percentage against the total number of isolates.

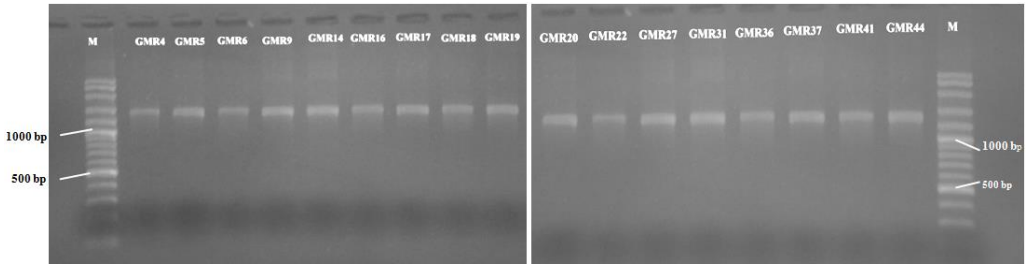

Figure 1: Purified PCR product of the 16S rRNA gene of 17 antifungal-producing *Streptomyces* strains.

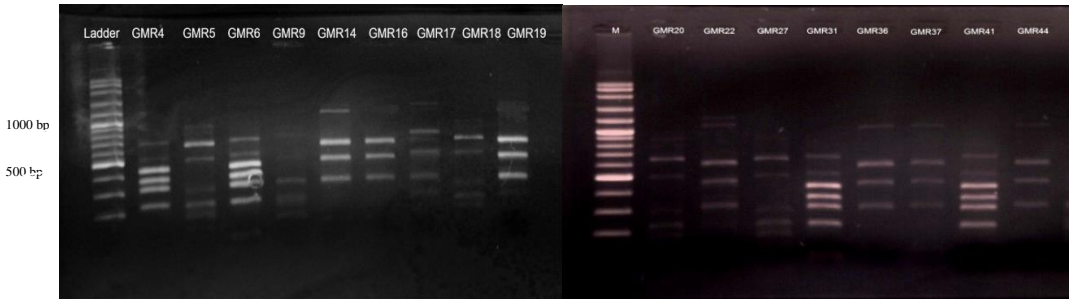

Figure 2: The ARDRA pattern of amplified 16S rRNA genes from 17 antifungal-producing *Streptomyces* strains following digestion by the restriction enzyme *Hae*III.

Diversity of strains assessed by ARDRA profiling

Amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) was applied to the 17 isolates that showed antifungal activity. The PCR products of 16S rRNA genes from actinomycetes were each about 1.3 kbp long (Figure 1). The purified amplicon products were digested with the restriction enzyme *Hae*III for fingerprinting analysis (Figure 2).

The main digestion products of the gene were 300-500 bp in size. Based on the digestion pattern, the *Streptomyces* spp. strains were assigned to eight different groups, namely group I harboring one strains (GMR20); group II harboring one isolate (GMR31 and GMR41); group III harboring one strains (17); group IV harboring one strains (GMR18); group V harboring three isolates (GMR36, GMR37, and GMR44); group VI harboring six strains (GMR5, GMR14, GMR16, GMR19, GMR22, and GMR27); group VII harboring one strain (GMR9), and groups VIII harboring two strains (GMR4, and GMR6) (Figure 3).

ARDRA analysis of the 16S rRNA gene is a rapid and inexpensive method that can be used to study the diversity of microbial communities. It may be possible to identify actinomycetes to the genus level using four restriction endonucleases even without sequencing (Zhang *et al*., 2006). The eight phylotypic groups derived from the ARDRA-based dendrogram in the present study revealed that actinomycetes in the cajuput plant soil rhizosphere were diverse. Group VI was the dominating group harboring six strains. However, the colony morphologies of all the strains isolated were different (data not shown). Strains of *Streptomyces* were difficult to partition at the subgeneric level although some of them

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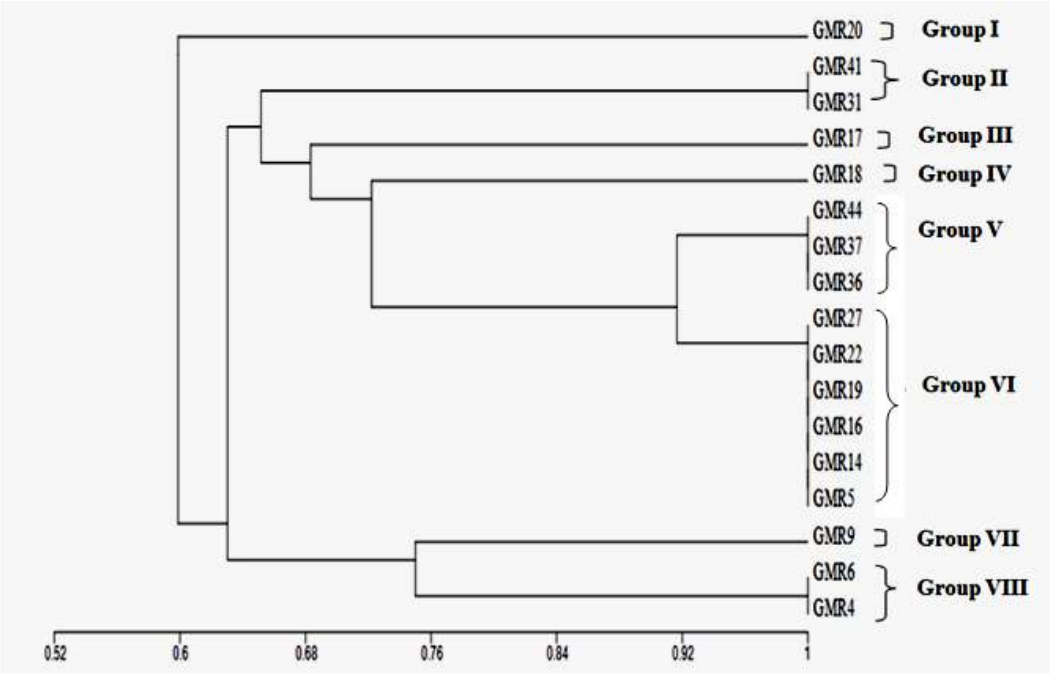

Figure 3: Dendrogram showing relationships between 17 representative strains of *Streptomyces* based on the UPGMA analysis of the ARDRA profile following digestion by *Hae*III restriction enzyme.

Table 2: *In vitro* dual culture assay of antagonistic potency of *Streptomyces* isolates against various fungi.

No	Isolate code*	Inhibition against tested fungi (%)			
		S.cerevisiae BY 47420	*C.albicans* CGMCC 2538	*A. flavus* NRLL 3357	*F.oxysporum* KFCC 11363P
1	GMR-4	56.00±1,112	63,67±3,512	0	0
2	GMR-5	61,67±1,527	68.00±4,359	0	44.00±0,121
3	GMR-6	64.00±1,020	69.00±2,887	59,67±3,215	65,67±5,507
4	GMR-9	66.00±5,131	60,66±6,110	62.00±6,083	64.00±2,199
5	GMR-14	58.01±1,117	60.00±2,646	66.00±3,606	60.00±2,645
6	GMR-16	73.00±4.933	66.00±13.011	57.67±3.512	60.00±3.467
7	GMR-17	64.00±7.810	56.00±3.464	55.33±0.577	61.00±4.358
8	GMR-18	65.67±2.082	67.67±4.726	60.00±2.646	60.67±3.214
9	GMR-19	60.00±7.211	65.00±2.121	54.00±1.732	58.00±3.464
10	GMR-20	50.01±13.115	58.00±2.646	58.00±3.11	70.01±2.645
11	GMR-22	88.00±1.024	80.01±2.636	60.01±2.623	84.67±2.081
12	GMR-27	58.00±2.057	52.00±2.009	52.00±2.647	58.00±7.937
13	GMR-31	44.00±3.606	0	44.00±4.583	62.00±17.435
14	GMR-36	60.00±4.359	64.00±5.292	48.00±2.646	59.00±17.434
15	GMR-37	55.00±1.002	66.67±10.599	52.00±3.099	60.33±2.082
16	GMR-41	61.67±8.145	64.00±3.215	54.00±2.646	57.67±6.027
17	GMR-44	57.33±2.082	63.00±3.091	60.00±8.888	60.33±3.217

* Coding system of antifungal producing of *Streptomyces* spp.
Average ± standard error from triplicate samples. Strong inhibition (>80%), moderate inhibition (<80>50%), weak inhibition (<50%).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(7) 2018, pp. 663-673
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could be distinguished from the others (Cook and Meyers, 2003). 16S rRNA gene fingerprinting analysis can effectively reduce the number of strains required for sequencing while screening for diversity. In this study, we made use of one restriction enzyme, *Hae*III, while different ARDRA patterns allowed us to differentiate effectively the strains into distinct groups or genera up to an extent.

Table 3: *In vitro* agar block assay of a selected strain (GMR22) for inhibition of several test microbes.

Test microbes	Diameter of inhibition zone (mm) of the selected strain (GMR22)
Yeasts	
C. albicans CGMCC 2538	35.64±0.86[b]
S. cerevisiae BY 47420	34.87±0.75[b]
Bacteria	
Staphylococcus aureus ATCC 25923	37.66±0.74[a]
S. mutan	31.34±1.02[c]
Pseudomonas aeruginosa	31.54±0.32[c]
Shigella dysentriae	39.32±0.77[a]
Bacillus subtilis ATCC 6633	32.67±1.17[c]
Escherichia coli ATCC 25922	34.13±1.10[b]

*Values in the table are means of three independent experiments and error bars indicate standard deviations of the means. Letter: a, b, c, show significant difference using LSD test (p>0.05).

Evaluation of antifungal-producing strains

In earlier work, actinomycetes that were isolated from mangrove ecosystem were shown to be capable of inhibiting bacterial and fungal pathogens activities (Das *et al*., 2014). *S. lydicus, S. lavendulae, S. albus, S. antibioticus, S. diastaticus,* and *S. phaeochromogenes* displayed varying degrees of antibacterial and antifungi activities (Sunanda Kumari *et al*., 2009).

In the present study, the isolated bacterial strains were deemed to be antifungal-producing if growth of mycelium in the test plates of the dual culture assays was inhibited as compared to that in the controls. The results showed that all the strains isolated inhibited the various test fungi by more than 50%, except strains GMR4, GMR5, and GMR31 against *A. flavus, F. oxysporum* and *C.albicans*, respectively. Three strains, GMR9, GMR18, and GMR22, showed at least 60% inhibition against all tested fungi. However, high inhibition was observed only with the unicellular fungi (the yeasts) (Table 2). The results also showed that antifungal activity of the strain GMR22 was high (60-80%) against both unicellular and filamentous fungi. This strain was hence selected for further characterization and analyzed for antifungal compound production (Figure 4). In addition to the fungi tested, the strain also exhibited inhibitory action against several bacteria such as *S. aureus, P. aeruginosa, S. mutant*, and *Salmonella typhi* based on agar block assay (Table 3).

Actinomycetes are microbes that are known for their ability to colonise roots and to provide numerous benefits, both directly and indirectly, to plants. Rhizospheric-actinomycetes can utilise sugars commonly found in plant root rhizosphere. At the same time, these microbes produce exudates that promote plant growth (El-Tarabily, 2008), and that act as siderophores (Cao *et al*., 2005) or biocontrol substances (Errakhi *et al*., 2007; Eccleston *et al*., 2010; Wang *et al*., 2013). The capability of the isolates to inhibit fungi could be achieved through several mechanisms, including the inhibition of cell wall and ergosterol metabolism, or the inhibition of DNA synthesis (Hooper, 2001).

Detection of antifungi substance of the selected strain (GMR22)

While antifungal compounds from actinomycetes could also be effectively extracted with ethylacetate (Augustine *et al*. 2005), further investigations on the component responsible for antifungal activities were performed on a chloroform extract in the present study. Following fractionation on a chromatography column, the active fraction was subjected to TLC-bioautography (Rios *et al*., 1988), the profile of which featured three spots. One of the spots (R_f 0.42) showed antifungal activity as indicated by a clear zone surrounding the spot (Figure 5).

Morphological and cultural characterization of the selected strain (GMR22)

One of the 17 antifungal-producing strains, GMR22, was analysed by morphological and cultural characterization according to the protocols of the International *Streptomyces* Project (ISP). Spores of the isolate were examined under the scanning electron microscope (SEM). Morphological characters of the strain included spiral spore chains, ornaments and warty spores. Sclerotic granules and sporangia were not observed. The aerial spores were grey, but there was no production of melanin when cultured on tyrosine media.

The cell wall hydrolysate contained LL-diaminopimelic acid (LL-DAP); a sugar pattern could not be detected. Morphological observations on a 2-week-old culture of the strain GMR22 grown on ISP2 medium revealed the typical characteristics of the genus *Streptomyces*. Scanning electron microscopy showed the presence of extensively branched substrate mycelia with chains forming rugose ornamented spores in spiral spore chains (Figure 6). On developed on the substrate with aerial mycelia present, but development on nutrient agar was poor. The aerial and substrate mycelium colour was determined on media ISP3. NaCl. The strain was observed to grow in the presence of 0 to 5% NaCl. As Larsen (1986) notes,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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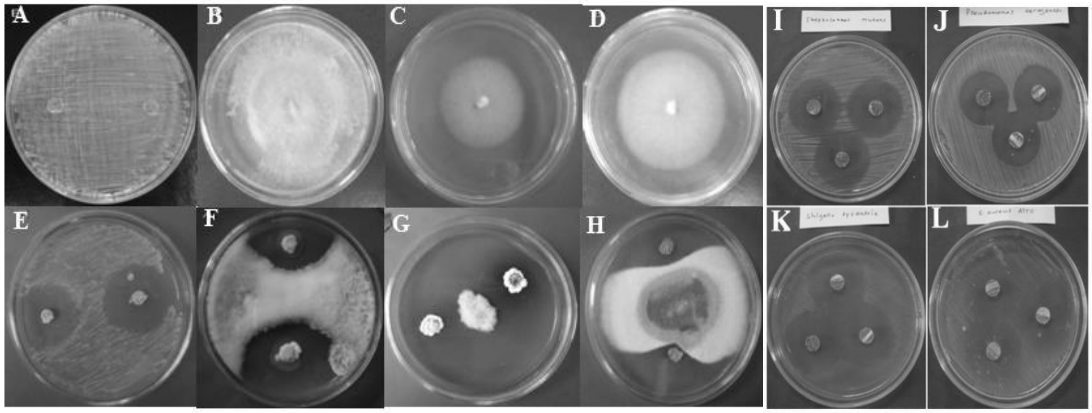

Figure 4: Representation of antagonistic activities (agar block assay) of the strain GMR22 against the tested fungi. (A-D) controls, (E-H) corresponding tested fungi, (E) *C. albicans* CGMCC2538, (F) *Trichoderma resei* NBRC 31329, (G) *Fusarium oxysporum* KFCC 11363P, (H) *Aspergillus flavus* NRLL 3357. GMR22 also inhibited the growth of bacteria such as (I) *Streptococcus mutan*, (J) *Pseudomonas aeruginosa,* (K) *Shigella dysentriae,* (L) *Staphylococcus aureus* ATCC 25923.

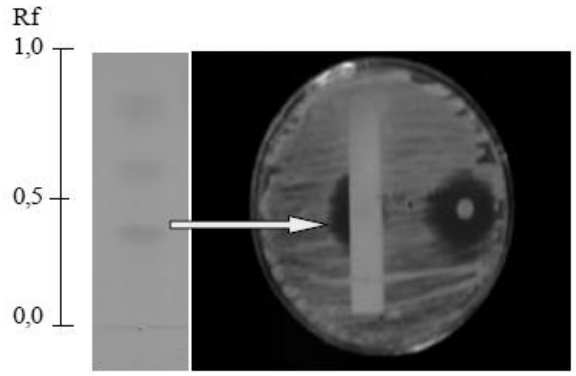

Figure 5: TLC-bioautography profile of antifungal activity of spot at R_f 0.42 (arrow). The mobile phase used was [$CHCl_3$·MeOH, 20:1(v/v].

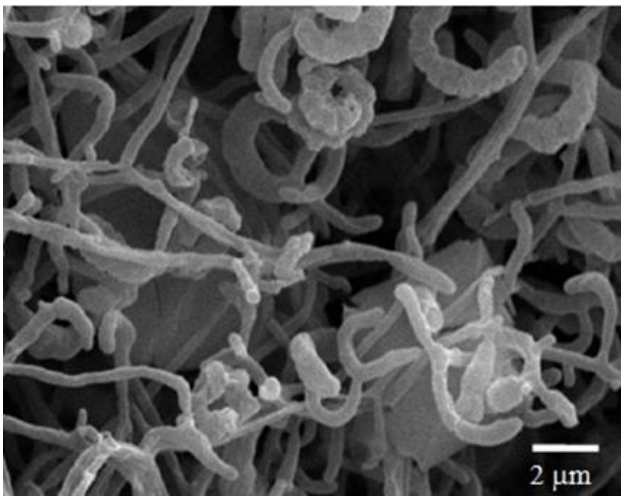

Figure 6: Morphological ultrastructure under the scanning electron microscope showing spore ornamentation of the strain GMR22. Scale bar: 2 µm.

acetomycetes can be grouped according to their NaCl tolerance, i.e. slight (2 to 3%), moderate (5 to 20%) and extreme (higher than 12%).The strain was inhibited by several types and concentrations of antibiotics, but not by sulphanilamide (75 µg).

Based on the physiological and biochemical properties of the isolated strain, there is strong evidence to indicate that GMR22 belongs to the genus *Streptomyces*. To confirm this, the 16S rRNA gene sequence obtained was subjected to GenBank BLAST search analyses. The phylogenetic tree shows that GMR22 is clustered in a clade of species found in Southeast Asia, especially in Indonesia and Malaysia. Members of the clade include *S. asiaticus* AB249947, *S. cangkringensis* AJ391831, *S. indonesiensis* DQ334783, *S. yogyakartensis* AB249942, and *S. malaysiensis* AB249918 (Figure 7). Based on the bootstrap value of 99%, strain GMR22 may be identified as a strain bearing close similarity to *S. vayuensis*. Sequence analysis of its 16S rRNA gene showed that strain was closely related to *S. vayuensis* and *S. malaysiensis.* Janda and Abbott (2007) opine that identification of a microbial strain to the species level is possible where similarity of the 16S rRNA gene sequence is at least 99% compared to that of the references type. However, differences were observed in morphology and spore chain formation, spore ornament and pigment diffusion when GMR22 was compared with *S. vayuensis* and *S. malaysiensis.* Spores of GMR22 were fragmented, whereas those of *S. vayuensis* and *S. malaysiensis* were not. Similarly, spore ornament and pigment diffusion of the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(7) 2018, pp. 663-673
DOI: http://dx.doi.org/10.21161/mjm.108617

latter two species were rugose and produced colour diffusion pigment; this was not the case with GMR22. Nevertheless, all three strains displayed grey to black aerial mycelium after prolonged incubation on starch agar media. Simammora *et al*. (2016) note that even when high molecular similarity with reference strains are observed, dissimilarities that show up in morphological characters are not uncommon.

Table 4: Morphological and physiological properties of the selected strain (GMR22).

Characteristic	Observation
Spore chain morphology	Closed spiral
Ornaments chain spore	Warty spore
Melanin production	-
Color of spore mass/substrate mycelium on Oatmeal agar	Grey/black
Utilization of carbon sources	
α Lactose	++
Xylan	++
D-Mannitol	++
Cellebiose	++
Meso-Erythritol	++
Raffinose	++
Melezitose	++
Arabinose	++
Trehalose	++
Utilization of nitrogen sources	
DL-α-amino-n-butyric acid	++
L-valine	++
L-asparagine	++
L-isoleusine	-
L-arginine	++
NH_4Cl	++
Melanin production	-
Color of soluble pigment	-
Growth at temperature (°C)	15 to 45
Growth at NaCl (%)	0 to 5
Growth at pH	5 to 9
Growt at antibiotic:	
Ampicillin (10µg)	-
Chloramphenicol (30µg)	-
Erythromycin (15µg)	-
Neomycin (30µg)	-
Penicillin G (10 IU)	-
Rifampicin (10µg)	-
Gentamycin (10µg)	-
Streptomycin (10µg)	-
Kanamycin (23µg)	-
Cycloserin (25 µg)	-
Novobiocin (10 µg)	-
Sulphanilamide (75 µg)	+

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(7) 2018, pp. 663-673
DOI: http://dx.doi.org/10.21161/mjm.108617

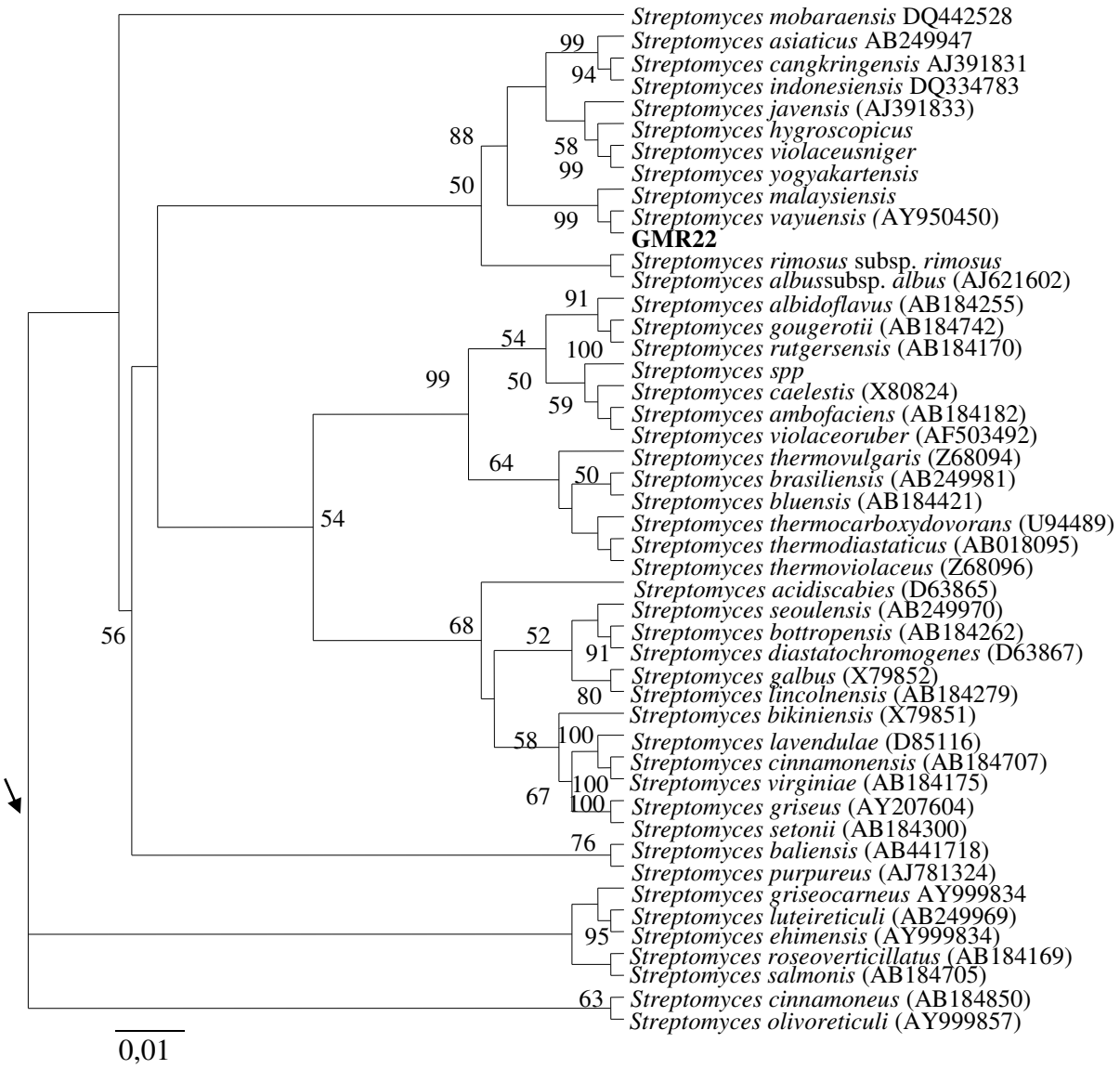

Figure 7: Neighbor-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing the relationships between strain GMR22 and members of the genus *Streptomyces*. The numbers at the nodes indicate the level of bootstrap support based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are shown. Scale bar indicates 0.1 substitutions per nucleotide position (Hahn *et al.*, 1999).

CONCLUSIONS

Our study revealed the abundance and diversity of *Streptomyces*-like antifungal producing microbes in the limestone soil rhizosphere of the cajuput plant. This rhizosphere represents a potential new source of actinomycetes that synthesize biologically active compounds. One selected strain described in the present study, GMR22, showed strong potency in the antifungal it produced and has the potential to be developed as a commercial biofungicide.

ACKNOWLEDGMENT

We would like to acknowledge the financial support given by the Ministry of Research and Technology, Higher Education of Indonesia, to this study under grant HIBAH DOKTOR 2012.

REFERENCES

Aghighi, S. G. H., Bonjar, S. and Saadoun, I. (2005). First report of antifungal properties of a new strain of

Streptomyces plicatus (strain 101) against four Iranian phytopathogenic isolates of *Verticillium dahliae*, a new horizon in biocontrol agents. *Biotechnology* **3, 90-97.**

Atalan, E., Manfio, G. P., Ward, A. C., Kroppenstedt, R. M. and Goodfellow, M. (2000). Biosystematic studies on novel *Streptomycetes* from soil. *Antonie Van Leeuwenhoek International Journal* **77, 337–353.**

Augustine, S. K., Bhavsar, S. P. and Kapadnis, B. P. (2005). A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23. *Journal of Biosciences* **30, 201-211.**

Ayuoso, A. S. and Genilloud, O. (2005). New PCR primers for the screening of NRPS and PKS-I systems in *Actinomycetes*: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microbial Ecology* **49, 10-24.**

Badji, B., Zitouni, A., Mathieu, F., Lebrihi, A. and Sabaou, N. (2006). Antimicrobial compounds produced by *Actinomadura* sp AC104 isolated from an Algerian Sahara soil. *Canadian Journal of Microbiology* **55, 373-382.**

Barakate, M., Ouhdouch, Y., Oufdou, K. H. and Beaulieu, C. (2002). Characterization of rhizosperic soil *Streptomyces* from Moroccan habitats and their antimicrobial activities. *World Journal of Microbiology and Biotechnology* **18, 49-54.**

Basil, A. J., Strap, J. L., Heather, M., Smith, K. and Crowford, D. L. (2004). Studies on the microbial populations of the rhizosphere of big sagebrush (*Artemisia tridentata*). *Journal of Industrial Microbiology and Biotechnology* **31, 278-288.**

Bouizgarne, B., Hadrami, I. E. and Ouhdouch, Y. (2006). Novel production of isochainin by a strain *Streptomyces* sp. isolated from rhizosphere soil of the indigenous Moroccan plant *Argania spinosa* L. *World Journal of Microbiology and Biotechnology* **22, 423-429.**

Cao, L., Qiu, Z., You, J., Tan, H. and Zhou, S. (2005). Isolation and characterization of endophytic streptomycete antagonistics of fusarium wilt pathogen from surface-sterilized banana roots. *FEMS Microbiology Letters* **247, 147-152.**

Cook, A. E. and Meyers, P. R. (2003). Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *International Journal of Systematic and Evolutionary Microbiology* **53, 1907-15.**

Costa, F. G., Zucchi, T. D. and de Melo, I. S. (2013). Biological control of phytopathogenic fungi by endophytic actinomycetes isolated from maize (*Zea mays* L.). *Brazilian Archive of Biology and Technology* **56, 948-955.**

Crawford, D. L., Lunch, J. M., Whipps, J. M. and Ousley, M. A. (1993). Isolation and characterization of actinomycetes antagonists of fungal root pathogen. *Applied and Environmental Microbiology* **59, 3899-3905.**

Das, A., Bhattacharya, S., Mohammed, A. Y. H. and Rajan, S. S. (2014). *In vitro* antimicrobial activity and characterization of mangrove isolates of streptomycetes effective against bacteria and fungi of nosocomial origin. *Brazilian Archive of Biology and Technology* **57, 349-356.**

Doumbou, C. L., Hamby-Salove, M. K., Crawford, D. L. and Beaulieu, C. (2002). Actinomycetes, promising tools to control plant diseases and to promote plant growth. *Phytoprotection,* **82, 85-102.**

Eccleston, K. L., Brook, P. R. and Kurtböke, I. (2010). Assessment of the role of local strawberry rhizosphere-associated *Streptomyces* on the bacterially-induced growth and *Botrytis cinerea* infection resistance of the fruit. *Sustainability* **2, 3831-3845.**

El-Tarabily, K. A. (2008). Promotion of tomato (*Lycopersicon esculentum* Mill.) plant growth by rhizosphere competent 1-aminocyclopropane-1-carboxylic acid deaminase-producing streptomycete actinomycetes. *Plant and Soil* **308, 161-174.**

Errakhi, R., Bouteau, F., Lebrihi, A. and Barakate, M. (2007). Evidences of biological control capacities of *Streptomyces* spp. against *Sclerotium rolfsii* responsible for damping-off disease in sugar beet (*Beta vulgaris* L.) *World Journal of Microbiology and Biotechnology* **23, 1503-1509.**

Frandberg, E., Peterson, C. Lundgren, L. N. and Schnurer J. (2000). *Streptomyces halstedii* K122 produces the antifungal compounds bacilomycin B1 and C1. *Canadian Journal of Microbiology* **46, 753-758.**

Franco-Correa, M., Quintana, A., Duque, C., Suarez, C., Rodriguez, M. X. and Barea, J. M. (2010). Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. *Applied Soil Ecology* **45, 209-217.**

Garland, J. L. (1996). Patterns of potential C source utilization by rhizosphere communities. *Soil Biology and Biochemistry* **36, 489- 498.**

Grayston, S. J., Wang, S. Q., Campbell, C. D. and Edwards, A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* **30, 369- 378.**

Getha, K. and Vikinesway, S. (2005). Evaluation of *Streptomyces* sp. strain g10 for suppression of Fusarium wilt and rhizosphere colonization in pot-grown banana plantlets. *Journal of Industrial Microbiology and Biotechnology* **32, 24-32.**

Goodfellow, M. and T. Cross. (1984). Classification. In: The Biology of the Actinomycetes. Goodfellow, M., Mordarski, M. and Williams, S. T. (eds.). Academic press, U. K. **pp. 7-164.**

Hahn, D., Nickel, A. and Dawson, J. (1999). Assessing Frankia populations in plants and soil using molecular methods. *FEMS Microbiology Ecology* **29, 215-227.**

Hooper, D. C. (2001). Mechanisms of action of antimicrobials: Focus on fluoroquinolones. *Clinical Infectious Diseases* **32 (Suppl1), 9-15.**

Janda, J. M. and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic

laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology* **45, 2761-2764.**

Khamna, S., Yokota, A. and Lumyong, S. (2009). Actinomycetes isolated from medicinal plant rhizosphere soils: Diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. *World Journal of Microbiology and Biotechnology* **25, 649-655.**

Kloepper, J. W. and Schroth, M. N. (1981). Relationship of *in vitro* antibiosis of plant growth promoting rhizobacteria to plant growth and the displacement of root microflora. *Phytopathology* **71, 1020-1024.**

Larsen, H. (1986). Halophilic and halotolerant microorganism: An overview historical perspective. *FEMS Microbiology Reviews* **39, 3-7.**

Lee, J. P. and Hwang, B. K. (2002). Diversity of antifungal actinomycetes in various vegetatif soils of Korea. *Canadian Jouranl of Microbiology* **48, 407-417.**

Loqman, S., Barka, E. A., Clement, C. and Ouhdouch, Y. (2009). Antagonistic actinomycetes from Moroccan soil to control the grapevine gray mold. *World Journal of Microbiology and Biotechnology* **25, 81-91.**

Lynch, J. M. (1983). Soil Biotechnology: Microbiological factors in crop productivity. Blackwell Scientific Publication, London. **pp. 191.**

Macdonald, L. M., Paterson, E., Dawson, L. A. and McDonald, A. J. S. (2004). Short-term effects of defoliation on the soil microbial community associated with two contrasting *Lolium perenne* cultivars. *Soil Biology and Biochemistry* **36, 489-498.**

Nishimura, T., Meguro, A., Hasegawa, S., Nakagawa, Y., Shimizu, M. and Hunoh, H. (2002). An endophytic actinomycete, *Streptomyces sp*. AOK-30, isolated from Mountain Laurel and its antifungal activity. *Journal of General Plant Pathology* **68, 390-397.**

Pandey, A. and Palni, L. M. (2007). The rhizosphere effect in trees of the Indian Centra Himalaya with special reference to altitude. *Applied Ecology and Environment Research* **5, 93-102.**

Petrosyan, P., Garcia-Verela, M., Luz-Madrigal, A., Eluitron, C. and Flores, M. E. (2003). *Streptomyces mexicanus* sp. nov., a xylanolytic microorganism isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* **53, 269-273.**

Rios, J. L., Recio, M. C. and Villar, A. (1988). Screening methods for natural products with antimicrobial activity: A review of the literature. *Journal of Ethnopharmacology* **23, 127-149.**

Rosruen, T. and Pornpakaku, S. (2008). Screening for antimicrobial from endophytic fungi isolated from *Melaleuca cajuputi* Powell. Annual Meeting and International Conference of the Thai Society for Biotechnology, October 14th-17th. **p. 374-381**

Sacido, A. A. and Genilloud, O. (2004). New PCR Primers for the screening of *nrps* and *pks-I* systems in actinomycetes: Detection and distribution of these biosynthetic gene sequences in major taxonomy groups. *Microbial Ecology* **49, 10-24.**

Sembiring, L. (2009). Molecular phylogenetic classification of Streptomycetes isolated from the rhizosphere of tropical legume (*Paraserianthes falcataria*) (L.) Nielsen. *Hayati Jurnal Biosains* **16, 100-108.**

Shirling, E. B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology* **13, 313-340.**

Simamora, C. J. K., Solihi, D. D. and Lestari, Y. (2016). Culturable and unculturable actinomycetes assosiated with the sponge Neofibularia from Bira Island, Indonesia. *Malaysian Journal of Microbiology* **12, 211-220.**

Söderberg, K. H. and Bååth, E. (1998). Bacterial activity along a young barley root measured by the thymidine and leucine incorporation techniques. *Soil Biology and Biochemistry* **30, 1259-1268.**

Sunanda Kumari, K., Uma Devi, K. and Apparao, A. (2009). Characterization of marine *Streptomyces* from Visakhapatnam coast. *Drug Invention Today* **1, 78-80.**

Suzuki, S., Yamamoto, K., Okuda, T., Nishio, M., Nakanishi, N. and Komatsubara, S. (1999). Selective isolation and distribution of *Actinomadura rugatobiospora*. strains in soil. *Actinomycetologica* **14, 27-33**.

Suzuki, I., Okuda, T. and Komatsubara, S. (2000). Selective isolation and distribution of *Actinobispora* strain in soil. *Canadian Journal of Microbiology* **46, 708-715.**

Taechowisan, T. and Lumyong, S. (2003). Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galena* against phytopathogenic fungi. *Annual of Microbiology* **53, 291-298.**

Walker, T. S., Bais, H. P., Grotewold, E. and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiology* **132, 44-51.**

Wang, Z., Wang, C., Li, F., Li, Z., Chen, M. and Wang Y. (2013). Fumigant activity of volatiles from *Streptomyces alboflavus* TD-1 against *Fusarium moniliforme* Sheldon. *Journal of Microbiology* **51, 477-483.**

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., Wang, Y., Huang, Y. and Cui, H. (2013). Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiology Letters* **341, 45-51.**

Xu, L. H., Li, Q. R. and Jiang, C. L. (1996). Diversity of soil actinomycetes in Yunnan, China. *Applied and Environmental Microbiology* **62, 244-248.**

Zhang, H., Lee, Y. K., Zhang, W. and Lee, H. K. (2006). Culturable actinobacteria from the marine sponge Hymeniacidon perleve: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie Van Leeuwenhoek International Journal* **90,159-169.**

Zhu, H., Swierstra, J., Wu, C., Girard, G., Choi, Y. H., van Wamel, W., Sandiford, S. K. and van Wezel, G. P. (2014). Eliciting antibiotics active against the ESKAPE pathogens in a collection of actinomycetes isolated from mountain soils. *Microbiology* **160, 1714-1725.**