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Contributed Paper

The Antagonistic Activity of Bioactive Compound Producing *Streptomyces* of Fusarium Wilt Disease and Sheath Blight Disease in Rice

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ABSTRACT

Rhizospheric actinomycetes were screened for bioactive compound production such as antibiotic, siderophore and cell wall degrading enzyme against Fusarium wilt disease and sheath blight disease in rice which are caused by *Fusarium oxysporum* and *Rhizoctonia solani* AG-2, respectively. A total of 150 strains was classified based on their cultural and physiological characterization such as color of aerial mycelium, color of substrate mycelium and spore chain morphology combined with cell wall composition analysis. They were classified as *Streptomyces* (52.7 %), non-*Streptomyces* (35.3 %) and unidentified (12 %). Involvement of cell wall degrading enzymes such as chitinase, cellulase and β-1,3-glucanase was investigated. Results showed that isolate RHI-43 produced high levels of chitinase (3.50 U/ml), cellulase (4.90 U/ml) and β-1,3-glucanase (0.45 U/ml) while isolate RHI-114 produced high levels of protease (15 U/ml). On the basis of dual culture assays, isolate RHI-39 and isolate RHI-146 were selected on the basis of broad spectrum activities and identified using cell wall composition analysis combined with 16S rDNA analysis and phylogenic classification. These isolates were identified as *Streptomyces lydicus* (for isolate RHI-39) and *Streptomyces corchorussi* (for isolate RHI-146). Further, culture filtrates of isolate RHI-146 inhibited the growth of all fungi tested which showed hyphal swelling and abnormal shapes of tested fungi under microscope. These results suggest that *Streptomyces* isolate RHI-39 and *Streptomyces* isolate RHI-146 may be utilized as an environmental friendly biocontrol agent against some important rice-pathogenic fungi *in vitro*.

Keywords: biological control, fusarium wilt disease, sheath blight disease, bioactive compound, *Streptomyces*

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a major food crop of Asia. Rice diseases are caused by different groups of pathogen such as fungi, viruses, bacteria and nematodes [1]. Fusarium wilt disease and sheath blight disease are caused by *Fusarium oxysporum* and *Rhizoctonia solani*,

respectively, which lead to high losses of rice in many countries of Asia. *Fusarium oxysporum* enters the plant from young roots and blocks water and nutrient transportation in the vessels *via* macro and micro conidia. The fungus causes leaf chlorosis and slight vein clearing on outer leaflets, followed by wilting and dropping of leaves, then xylem browning of stem and finally death of the above ground parts [2]. There are no effective, economical and practical ways to control Fusarium wilt disease due to re-colonization of air-borne spores of *Fusarium oxysporum* under favorable conditions [2]. *Rhizoctonia solani* attacks rice at maximum till ring stage by sclerotium over winter in soil and plant debris and causes poor filling of the grains and emergence of panicles [3]. Control of both pathogens is difficult because of their ecological behavior, their extreme broad host range and the high survival rate of fungal spore under various environmental conditions and no rice variety capable of resistant completely to these pathogen [4]. The most common approach to control rice disease is to use chemical fungicides but they can destroy the balance of ecosystems and the contamination by their toxic residues may be harmful to human and animals. Biological control may be effective in minimizing the incidence of fungal pathogens.

Streptomyces sp. have been explored over several decades for antibiotic production. In the last few years strong antimicrobial potential of *Streptomyces* has received attention for plant-growth promotion. *Streptomyces* degrade a broad type of biopolymers by secreting hydrolytic enzymes and effective colonizer of plant root systems and endure unfavorable conditions by forming spores. Actinobacteria produce a variety of antibiotics possessing polyketides, β-lactams, peptide and other secondary metabolites such as antifungal, antibacterial and anti-tumor compounds [5]. Thus, *Streptomyces* are recognized as antimicrobial agents which inhibit plant pathogens such as soil-borne fungi [6]. The mode of action includes antifungal and antibiotics production, competition for iron by siderophore production, cell wall degrading enzyme production such as chitinase and glucanase, phenylacetic and sodium acetate production [7]. Various groups of bioactive compounds were produced by *Streptomyces* such as macrolide, benzoquinones, aminoglycosides, polyenes and nucleoside antibiotics [8]. Application of *Streptomyces* as biocontrol agents is prolonging survival during commercial process and formulation after applying in the field. This is because *Streptomyces* are able to produce spores to maintain their viability at non-specific conditions [9]. Therefore, *Streptomyces* grow in a wide range of temperature which is aids the survival in the soil after application [9].

In this study, *Streptomyces* sp. were isolated from rice rhizosphere soils, and screened for antagonistic activities such as antibiotic production, siderophore production and hydrolytic enzyme activity against *Fusarium oxysporum*, and *Rhizoctonia solani* AG-2, the causal agents of Fusarium wilt disease and sheath blight disease in rice, respectively.

2. MATERIALS AND METHODOLOGY

2.1 Fungal Strains and Culture Conditions

Fusarium oxysporum and *Rhizoctonia solani* were isolated from root and leave of lowland rice in the north of Thailand and tested for pathogenicity. The virulent strains were grown on potato dextrose agar (PDA) and incubated at 28°C for 3-5 days. Stock cultures were maintained on PDA slants and stored at 4°C.

2.2 Isolation of Rhizospheric *Streptomyces*

Streptomyces strains were isolated from rice rhizospheric soil by serial dilution and spread plate technique. Briefly, soil sample (10 g) was suspended in 9 ml of basal salt solution (5.0 g/l KH_2PO_4 and 5.0 g/l NaCl), and the suspension was mixed using rotator shaker (150 rpm) at 28°C for 24 hr. Then, the dilution samples were spread on starch-casein-agar and incubated at 28°C for 2 weeks. After incubation, colonies of *Streptomyces* were selected on the basis of their morphological characteristics and purified onto ISP-2 agar [10]. To induce sporulation, purified colonies were subcultured on ISP-3 agar [10].

2.3 *Streptomyces* Screening for Antagonistic Activity Against Fusarium wilt Disease and Sheath Blight Disease

All strains were screened for their *in vitro* antagonism against *Fusarium oxysporum* and *Rhizoctonia solani* according to the method of Crawford *et al.*, (1993). Briefly, a 20 µl spore suspension of *Streptomyces* sp. (10^6 spore/ml) was spotted on one side of PDA plate and incubated at 28°C for 5 days. After incubation, mycelium's plugs of fungi (6.0 mm) were transfer to PDA plate and incubated at 28°C for 5 days. For control group, fungal plug was inoculated on PDA plate without *Streptomyces* sp. The radial fungal control and dual culture plates were measured at 5 days after incubation and levels of inhibition were calculated using the equation as previously mentioned by Yang and Crawford (1995) [11]. Briefly, R_1-$R_2/R_1 \times 100$, where R_1 is the radial distance (mm) grown by plant pathogenic fungi in direction of antagonist (a control value), and R_2 is the radial distance (mm) grown by fungal in dual culture. All strains were tested in triplicates.

2.4 Evaluation of the Parasitic Mechanism

The mycoparasitism of *Fusarium oxysporum* and *Rhizoctonia solani* cell wall by *Streptomyces* strain RHI-39 and *Streptomyces* strain RHI-146 was studied using light microscopy (CHS, Olympus optical Co. Ltd, Japan) and scanning electron microscopy (SEM) (JEOL JSM-6610CV, Japan). For scanning electron micrographs, the cultures on the dual culture plates were pre-fixed overnight in 2% $CH_2(CH_2CHO)_2$ in 0.1 M sodium cacodylate buffer pH 7.2-7.4 at 4°C; washed three times with the same buffer for 15 min; post-fixed at 4°C for 1h in 1% OsO4 in 0.1 M sodium cacodylate buffer; washed three times with 0.1 M sodium cacodylate buffer, pH 7.2-7.4 at 4°C for 15 min; dehydrated in a graded series of ethanol-amylacetate. Samples in the amyl acetate were critical point dried with Hitachi HCP-2 critical point dryer (HCP-2; Hitachi Co.Ltd., Japan); coated with Pt-Pd mixture for 4 min in Hitachi E-102 ion sputter (E-102; Hitachi Co. Ltd., Japan) and examined in a Hitachi S-510 SEM (JSM-6610 CV; Japan Electron Optics Laboratory Co. Ltd.).

2.5 Quantitative Determination of Cell Wall Degrading Enzymes

2.5.1 Assay for chitinase

Selected strains which show inhibitory effect on test fungi were grown in ISP-2 broth with continuous shaking at 150 rpm at 28°C for 10 days. After incubation, cell-free supernatant was collected by centrifugation at 8,000 rpm for 20 min at 4°C. Chitinase activity was determined by the reducing end group of N-acetylglucosamine (NAG) using DNS method [12].

2.5.2 Assay for cellulase

Cellulase activity was measured by spectrophotometric assay. A 500 ml aliquot of crude enzyme was incubated with 500 ml CMC (1% w/v-Carboxy Methyl Cellulose) in 0.1 M sodium phosphate buffer (pH 5.5). After incubation at 37°C for 60 min, the reaction was stopped by adding 2 ml of 3,5-dinitrosalicyclic acid and absorbance was measured at 530 nm in UV/Vis spectrophotometer. One Unit (U) is defined as the amount of enzyme that releases 1 mmol of reducing end group of glucose per minute [12].

2.5.3 Assay for β-1,3-glucanase

β-1,3-glucanase activity was measured according to the method of Singh *et al.*, (1996) [13] using laminarin from *Laminaria digitata* (Sigma USA) as substrate. Briefly, 500 l of 0.1% laminarin (in 50 mM citrate buffer pH 5.5) was mixed with 500 ml of culture supernatant. The mixture was incubated at 37°C for 60 min and the reaction was stopped with 2 ml of 3,5-dinitrosalicyclic acid [12]. The amount of reducing sugar released was measured at 530 nm in UV/Vis spectrophotometer. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that releases 1 mmol of glucose per min in these conditions.

2.5.4 Assay for protease

Five hundred ml of 0.5% casein in Tris-HCl buffer with 100 ml of enzyme solution were incubated at 37°C for 60 min. Reaction was stopped by adding 500 ml of 15% tricholoroacetic acid with shaking for 15 min. After incubation, the mixture was centrifuged at 3,000 rpm for 5 min at 40°C and one ml of supernatant was added with one ml of 1M NaOH and the amount of reducing sugar released was measured at 440 nm. Enzyme activity was calculated from standard curve of L-tyrosine and one unit of enzyme activity is equivalent to 1 mg of L-tyrosine min/ml under the assay conditions.

2.5.5 Siderophore production

The strains were assayed for the siderophore production on the Chrome Azurol S (CAS) agar according to the method of Alexander and Zuberrer (1991). CAS agar plates were prepared as followed. A dye made of CAS, Fe, and hexadecryl-trimethyl-ammonium bromide (HDTMA) was mixed with M9-based growth media. For 1 L of CAS-agar, 100 ml of CAS-Fe-HDTMA dye was mixed with 900 ml of prepared media. The CAS-Fe-HDTMA dye was prepared as followed; for 1 L, 10 ml of a 10 mM ferric chloride ($FeCl_3$) in 100 mM hydrochloric acid (HCl) solution was mixed with 590 ml of a 1 mM aqueous solution of CAS. The Fe-CAS solution was added to 400 ml of a 2 mM aqueous solution of HDTMA. The resulting CAS-Fe-HDTMA solution was autoclaved for 25 min in a polycarbonate bottle that had previously been soaked overnight in 10 % (v/v) HCl then rinsed five times with Milli Q water. The CAS-Fe-HDTMA dye was stored at room temperature and covered from light until use.

The growth media was prepared; for 1 l of CAS-agar, 30.24 g of 1, 4-piperazine-diethanesulfonic acid (Pipes), together with 1 g of ammonium chloride (NH_4Cl), 3 g potassium phosphate (KH_2PO_4), and 20 g sodium chloride (NaCl) was dissolved into Milli Q water by adjusting the pH with 10M NaOH to 6.8. As a solidifying agent, 9 g of agar noble (Difco), agar or agarose was added to the solution. The volume was adjusted to 860 ml and the solution was autoclaved. After cooling, 30 ml of a sterile 10 % (w/v)

casamino acid (Difco) aqueous solution and 10 ml of a sterile 20 % (w/v) glucose aqueous solution were added. Finally, the 100 ml of CAS-Fe-HDTMA were added to the growth media. The final concentrations of the CAS agar components were as followed: 100 mM Pipes, 18 mM NH_4Cl, 22 mM KH_2PO_4, 2 % (w/v) NaCl, 0.3 % casamino acids, 0.2 % (w/v) glucose, 10 M $FeCl_3$, 58 mM CAS, and 80 mM HDTMA. All chemicals were obtained from Sigma-Aldrich.

CAS agar was spot inoculated with actinobacterial strain and incubated at 28°C for 7 days. When the actinomycetes consumed iron, they were present in the blue-colored CAS media. The colonies producing yellow to orange halos were considered positive for siderophore production.

2.6 *Streptomyces* Identification

2.6.1 Primary identification

2.6.1.1 Cultural and physiological characteristics

The genetic diversities of actinomycetes isolated from rhizospheric soil were classified using Bergey's Manual of Systemic Bacteriology [14]. The arrangement of spores was examined microscopically using a cover slip-implanting technique. The cover slip was implanted on ISP-2 agar and incubated at 28°C for 14 days, which allowed aerial mycelia to grow on the cover slip. The cover slip was removed from the culture plate and the mycelia were stained with crystal-violet dye for 5 min. The fixed mycelia were observed under a light microscope.

Taxonomic properties of isolate RHI-39 and isolate RHI-146 were evaluated by the methods of the international *Streptomyces* project (ISP) [14]. Morphology of the mature culture and color was observed on yeast extract-malt extract agar (ISP-2), inorganic salts-starch agar (ISP-4) and glycerol-asparagine agar (ISP-5). Melanin production was determined on peptone-yeast extract-iron agar (ISP-6), tyrosine agar (ISP-7) and tryptone-yeast extract broth (ISP-1), while the carbon utilization was carried out in carbon utilizing medium (ISP-9) with the addition of one of the following sugars; D-glucose (positive control), L-arabinose, sucrose, D-xylose, Inositol, D-mannitol, D-fructose, D-sorbitol, cellubiose and in the absence of a carbon source (negative control). The colony morphology of the actinomycetes grown was observed under a stereomicroscope (Olympus CH30, Japan).

2.6.1.2 Color determination

Aerial mass color on ISP-3 and ISP-4, substrate mycelia color and diffusible soluble pigments on ISP-5, melanin production on ISP-6 and ISP-7 were observed at 28°C for 14 days using a reference color key [14].

2.6.1.3 Cell wall composition analysis

Analysis of 2,6-diaminopimelic acid (DAP) was carried out to determine the cell wall chemotype. The isomeric type of DAP with the colony morphology was used to divide non-*Streptomyces* and unidentified genera from *Streptomyces* group. The unidentified genera were determined according to the limit formation of aerial mycelia and spores on the agar media used. In order to determine the genus of antagonistic actinomycetes isolate RHI-39 and isolate RHI-146, the 2,6- diaminopimelic acid, one of the cell wall components of actinomycetes mycelia was analyzed using the method of the ISP as described by Shirling and Gottlieb (1966) [15] and Bergey's Manual of Systemic Bacteriology [14]. Potential actinomycetes were cultured in ISP-2 medium (4.0 g yeast extract, 10.0 g malt extract, 4.0 g dextrose, 20.0 g agar and 1 L sterile water, pH 7.3) at 150 rpm, 28°C for

14 days. After cultivation, the culture broth was centrifuged to collect cell debris and cell debris were hydrolyzed with 6 N HCl and heated at 70°C for 18 h in water bath. The hydrolysate was filtrated with Whatman No.1 filter paper and evaporated to dryness in order to remove the residue HCl. Residue was dissolved in 1 ml of distilled water and applied onto TLC plate (15 × 20 cm, Merck Co., USA). Twenty microliters of 0.01 M *DL*-DAP (Sigma Chemical Co., USA) containing *meso*- and *LL*-DAP isomers and amino acids (alanine, glycine and glutamate) were loaded on the plates as a standard [15].

2.6.2 Secondary identification

2.6.2.1 DNA preparation

Secondary identification was determined based on phylogenetic position. The genomic DNA was extracted from biomass. The extraction was performed following the manufacturer's instructions for the FavorPrep™ Tissue Genomic DNA Extraction Mini Sample Kit (FAVORGEN®, Taiwan). The selected actinomycetes were grown for 5 days at 28°C with agitation in 100 ml of ISP-2 medium. Biomass was harvested by centrifugation (8000 *g*, 10 min) and washed twice with double-distilled water. Two hundred milligrams of mycelia were used for DNA extraction as followed; the sample was dispersed in 800 µl of the aqueous lysis solution (100 mmol / l Tris-HCl, pH 7; 20 mmol/l EDTA; 250 mmol/l NaCl; 2 % m/v SDS; 1 mg/ml lysozyme). Five microliters of 950 mg/ml RNase solution was added, and the suspension was incubated at 37°C for 60 min. Ten microliters of a proteinase K solution (20 mg/ml) was added and the lysis solution was re-incubated at 65°C for 30 min. The lysate was extracted with an equal volume of phenol and centrifuged (8000 *g*, 10 min). The aqueous layer was re-extracted with phenol (50-50 % v/v), then by chloroform (50-50 % v/v). DNA was recovered from the aqueous phase by the addition of NaCl (150 mmol/l final concentration) and two volumes of cool 95% (v/v) ethanol period to centrifugation. The precipitated DNA was cleaned with 50 µl of TE buffer (10 mmol/l Tris-HCl, pH 7.4; 1 mmol / l EDTA, pH 8), and stored at 20°C. The quantities of DNA solutions were measured at 260 nm.

2.6.2.2 16S rDNA sequence analysis

The concentration and purification of DNA samples were evaluated by electrophoresis on a 1% (w/v) agarose gel and visualized by staining with ethidium bromide. *Taq* DNA polymerase and a pair of universal primers (forward primer 27 (5'-AGT TTG ATC CTG GCT CAG GAC GAA CG-3') and reverse primer 1,525 (5'-AGC CGG TCC CCC TGC AAG-3') were used. PCR was performed as followed ;PCR amplication was carried out in 20 µl reaction mixture containing 4 µl of lysed bacterial suspension, 1X PCR bovine serum albumin, 5 % dimethyl sulfoxide (DMSO), 100 µM dNTP and 1.4 U of *Taq* DNA polymerase. Amplification was performed with a cycler and initial denaturation (2 min at 94°C) was followed by 30 PCR cycles (94°C for 30s, 60°C for 30s and 72°C for 60s) and final extension at 72°C for 10 min. Amplified DNA was purified using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). Purified PCR products were sequenced using the facilities of First Base Laboratories, Malaysia. Nearly complete 16S rRNA gene sequences (approximately 1,500 bp) were determined and compared with the corresponding sequences available in the GenBank database (http://www.ncbi-nlm-nih.gov/) using the BLAST program. The 16S rDNA sequences of isolate RHI-39

and isolate RHI-146 were aligned using CLUSTAL W and compared to published sequences of species with standing in nomenclature available from GenBank databases using the BLASTN algorithm (http://blast.ncbi.nlm.nib.gov/Blast.cgi). The DNA sequences of the isolates were deposited in GenBank at the National Center for Biotechnology Information (NCBI). The phylogenetic tree of the 16S rDNA sequence was constructed by the neighbor-joining method using MEGA 4.0 software package [16]. In pairwise comparisons, sequence similarities were computed using PHYDIT program Version 1.0 (http://plaza.snu.ac.kr/jchun/phydit).

2.7 Production of Antifungal Metabolites

Secondary metabolites produced by *Streptomyces* isolates were extracted according to the method of Ellaiah *et al*., (2005) [17]. Pure cultures of these isolate were transferred into ISP-2 medium and incubated at 30°C for 1 week under agitation at 150 rpm. After incubation, the cultures were harvested using centrifugation at 8,000 × g, 4°C and biomass was separated from broth. Culture filtrates were extracted with ethyl acetate to dryness under vacuum to yield a crude residue and dissolved in 10 % DMSO. Crude extracts were used for antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani* by agar well diffusion method. DMSO extracted at a concentration of 100 µg/ml was added to each well as negative control. After incubation at 30°C for 48-72 hr, diameters of inhibition zones of the tested fungi were determined.

2.8 Effect of *Streptomyces* Crude Extract on Mycelium Morphology of Tested Fungi

The tested fungi were separately grown on PDA plate at 28°C for 3 days and mycelia were cultured on surface of microscope slides covering the layers of PDA containing 20 % (v/v) culture extract. Inoculated slides were incubated at 28°C in the dark for 3 days and fungal hyphae present on the slides were stained with lactophenol cotton blue (Fluka Switzerland). Subsequent changes in the morphology of young emerging hyphae were observed under light microscope.

2.9 Statistical Analyses

The data were analyzed by variance analysis (ANOVA), and the mean separation was achieved by the DUNCAN's multiple range tests. All numeric differences in the data were considered significantly different at the probability level of $p \leq 0.01$.

3. RESULTS

3.1 Isolation and Screening of Antagonistic *Streptomyces*

A total of 150 *Streptomyces* isolates was obtained from rice rhizospheric soil samples collected from northern parts of Thailand. Colonies of *Streptomyces* were picked on the basis of morphological characteristics and identified by their form of opaque, rough, non-spreading morphology embedded in agar medium. Color of substrate and aerial mycelia were observed.

3.2 Antifungal Activity of *Streptomyces*

In this report, *Streptomyces* were selected for three traits that were often associated with biocontrol agents, ability to hydrolyze cell wall and cell membrane of pathogens, and production of siderophore and active metabolites against *Fusarium oxysporum* and *Rhizoctonia solani* AG-2. Among 150 isolates, 101 isolates (63.7 %) showed antagonistic ability by inhibiting the growth of tested fungi and the actinomycete isolates. Of them, 22 isolates (14.6 %) showed siderophore and antifungal activity as presented in Table 1.

Percentages of antagonistic ability against *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 were 32 % and 40 % respectively. *Streptomyces* colony color series showed antagonistic activities against Fusarium wilt disease and sheath blight disease. Results showed that grey colony gave the highest inhibition (35 and 40 isolates), followed by brown (27 and 30 isolates), white (8 and 9 isolates), yellow (6 and 6 strains) and green colony (not detected and 1 isolate), respectively (Table 2). The screening results for biocontrol traits are depicted in Table 1. Only 14.6 % of 150 antagonistic actinomycetes were positive for siderophore production and antifungal activity. The siderophore production was detected in all potential actinomycete isolates, showing yellow or orange halo zone on CAS-blue agar with 10.7-30.3 mm, respectively. Isolate RHI-39 and isolate RHI-146 gave the highest value of antifungal activities. All isolates demonstrated at least one trait of biocontrol potentials (Table 1). Thus, isolate RHI-146 exhibited maximum inhibition against pathogenic fungi *Rhizoctonia solani* AG-2, followed by isolate RHI-39 (Figure 2). Isolate RHI-39 showed maximum inhibition against to *Fusarium oxysporum*, followed by isolate RHI-146 (Figure 1). Further, 40 isolates (26.7%) had strong antagonistic activity against *Fusarium oxysporum* and *Rhizoctonia solani* AG-2. Results indicated that *Streptomyces* were released extracellular diffusible metabolites which inhibited hyphal growth of pathogenic fungi.

Table 1. CAS assay for analysis of siderophore produced by broad spectrum *Streptomyces* isolate and antifungal activity.

Isolate	Color change on CAS agar	Zone width (mm)	% mycelia inhibition in PDA in dual culture assay	
			Fusarium oxysporum	*Rhizoctonia solani* AG-2
RHI-19	orange	15.7±1.2 b	42.2±0.7 e	80.0±2.2 c
RHI-27	orange	16.0±1.0 b	60.1±8.8 c	81.8±6.1 c
RHI-29	orange	12.3±1.5 c	21.8±0.2 g	42.7±3.3 f
RHI-33	orange	11.3±1.2 c	53.3±2.3 d	70.9±5.2 de
RHI-35	orange	10.0±1.0 d	54.7±2.3 d	78.8±3.5 d
RHI-39	yellow	29.0±1.0 a	80.1±4.4 a	83.4±0.3 c
RHI-40	yellow	28.3±3.2 a	48.5±1.3 e	82.0±4.8 c
RHI-42	yellow	23.0±1.0 ab	54.0±5.3 d	71.4±7.6 de
RHI-43	orange	17.7±1.5 b	33.8±4.6 f	70.8±1.4 de
RHI-55	orange	26.3±1.2 ab	59.2±3.2 c	86.9±3.6 b
RHI-62	yellow	15.3±2.1 b	270.0±9.1 b	67.5±1.2 e
RHI-67	orange	26.3±1.2 ab	70.0±9.1 b	85.7±1.2 b
RHI-72	yellow	27.3±2.5 ab	37.3±0.3 f	66.0±5.2 e
RHI-80	yellow	28.7±2.1 a	50.7±1.2 d	74.5±3.8 de
RHI-81	orange	13.7±0.6 c	62.3±0.3 c	85.6±3.4 b
RHI-91	yellow	11.3±0.6 c	45.8±3.6 e	80.3±1.1 c
RHI-99	orange	11.0±1.0 c	67.9±7.5 c	87.7±6.0 b
RHI-104	orange	11.3±0.6 c	31.5±0.5 f	78.6±2.2 d
RHI-107	orange	15.3±0.6 b	37.7±0.3 f	83.9±4.8 b
RHI-116	orange	12.7±1.5 c	33.2±3.7 f	81.6±2.6 c
RHI-145	orange	10.7±1.5 c	24.1±1.6 g	65.1±2.4 e
RHI-146	yellow	30.3±0.6 a	75.0±6.3 b	97.0±1.7 a

* Means with the same letter are not significantly different (LSD) according to ANOVA test ($p \leq 0.01$).

Table 2. Effect of *Streptomyces* colony color series on antagonistic activities against Fusarium wilt disease and sheath blight disease of rice.

Parameter	Color series								
	Grey	Brown	White	Yellow	Green	Black	Orange	No produced	Total
Number of isolate	51	45	15	13	5	3	16	3	150
Percentage of colony color	34	30	10	10	8.6	3.3	2	2	100
Number of active isolate	49	35	14	8	2	2	11	1	113
Number of active isolate against *Fusarium oxysporum*	35	27	8	6	ND	2	7	1	86
Number of active isolate against *Rhizoctonia solani* AG-2	40	30	9	6	1	2	5	1	94

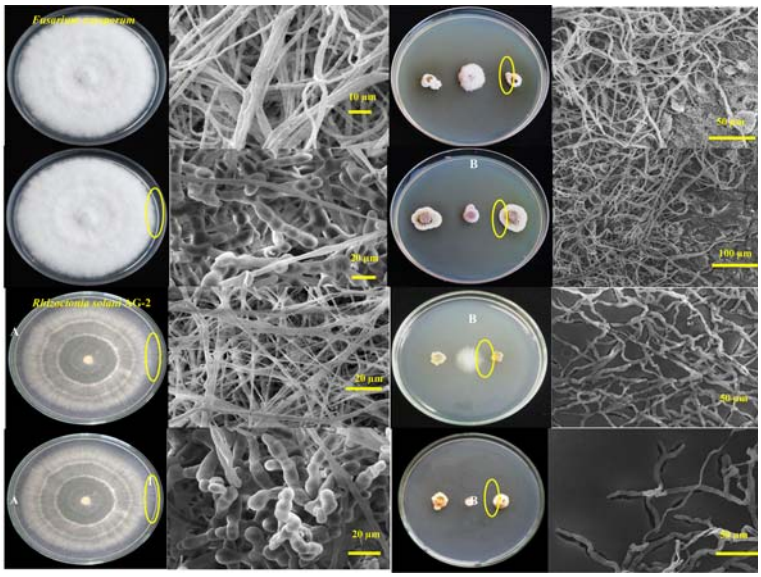

Figure 1. Scanning electron micrographs of the deformation of fungal mycelium of *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 by the antagonist at 5 days after co-culturing
1. *Streptomyces* isolate RHI-39
(A) mycelium of fungal pathogen (bar = 1 mM)
(B) small and thinner mycelium and abnormal branching of fungal pathogen caused by *Streptomyces* isolate RHI-39
2. *Streptomyces* isolate RHI-146
(A) mycelium of fungal pathogen (bar = 1 mM)
(B) small and thinner mycelium and abnormal branching of fungal pathogen caused by *Streptomyces* isolate RHI-146.

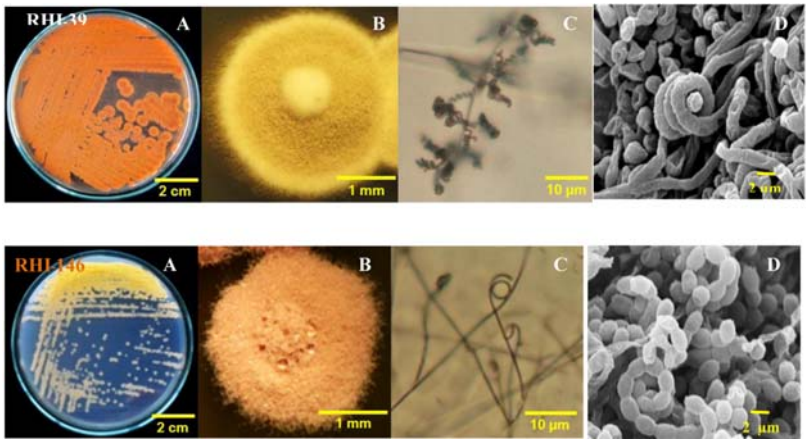

Figure 2. Morphology observation of *Streptomyces lydicus* isolate RHI-39 and *Streptomyces corchorussi* isolate RHI-146 on ISP-2 medium incubated at 30 °C for 7 days

(A) Color of substrate mycelium

(B) Color of aerial mycelium

(C) Aerial mycelium under light microscopy at 400× magnification

(D) Spores surface and spore chain under scanning electron microscopy at 10,000× magnification.

When growing *Streptomyces* isolate RHI-39 and *Streptomyces* isolate RHI-146 with *Fusarium oxysporum* and *Rhizoctonia solani* in PDA, the penetration of mycelium of *Streptomyces* isolate RHI-39 and *Streptomyces* isolate RHI-146 into the colony of *Fusarium oxysporum* were revealed under SEM (Figure 1). The area where both *Streptomyces* isolates grew had a clear inhibition zone. Thus, the clear inhibition zone was observed on the mycelia mass of *Fusarium oxysporum* indicating possible production of antibiological compounds and extracellular enzyme. The degradation of fungal mycelium by the antagonist was observed. The fungal mycelia were smaller and thinner which caused abnormal branching of hyphae and deformation of the fungi than control plate (Figure 1).

3.3 Production of Cell Wall Degrading Enzymes

Out of 150 *Streptomyces* isolates, 37 isolates produced chitinases (24.7 %), 79 isolates produced cellulase (52.7 %), 109 isolates produced protease (72.7 %), 56 isolates produced β-1,3-glucanase (62.7 %) and all isolates produced catalase (100 %). Screening of cellulase and β-1,3-glucanase producers were conducted by Congo red test as a preliminary study. After 5 days of incubation, 79 isolates showed clear zone on CMC agar which indicated cellulose degradation. Isolate RHI-41, RHI-42, RHI-43 showed maximum cellulase activity (4.25, 5.00, 4.84 U/ml). Further, 58 isolates produced β-1,3-glucanase (38.7 %) and glucanolytic activity were varied (0.03 to 2.53 U/ml). Moreover, 109 isolates exhibited protease activity (72.7 %) and isolates RHI-6, RHI-120, RHI-131 showed maximum protease activity (130, 112, 95 U/ml) (Table 3).

Table 3. Bioactive compounds and hydrolytic enzyme activity of potential *Streptomyces*.

Isolates	Hydrolytic enzyme activity (U/ml)			
	Cellulase	Chitinase	Protease	β-1,3-glucanase
RHI-6	2.25±0.60 e	ND	130±1.75 a	0.10±0.15 g
RHI-16	ND	3.58±1.25 a	ND	ND
RHI-23	0.90±0.65 h	ND	ND	ND
RHI-40	3.58±1.25 c	3.15±1.25 b	122±1.50 b	0.45±0.35 d
RHI-41	4.25±1.50 b	ND	ND	1.00±0.65 c
RHI-42	5.00±1.25 a	3.00±1.25 b	ND	2.50±1.25 a
RHI-43	4.84±1.35 ab	2.85±1.00 c	ND	ND
RHI-44	2.98±0.65 d	ND	ND	1.83±1.25 b
RHI-45	ND	ND	115±1.25 c	ND
RHI-57	2.87±1.25 d	ND	ND	0.10±0.35 g
RHI-58	0.95±0.65 h	ND	ND	0.06±0.25 h
RHI-60	1.32±0.45 g	ND	ND	0.36±0.25 e
RHI-72	1.45±0.6 f	2.53±0.65 d	95±0.65 d	0.30±0.15 f
RHI-80	0.87±1.25 h	0.87±0.35 fg	119±1.50 b	0.29±0.15 f
RHI-110	ND	0.95±0.15 f	60±0.35 f	ND
RHI-112	1.42±1.25 f	ND	ND	0.37±0.25 e
RHI-114	1.00±0.65 h	0.58±1.25 ef	85±0.45 e	0.04±0.15 h
RHI-120	ND	ND	112±1.00 c	ND
RHI-128	ND	1.79±1.00 e	ND	ND
RHI-149	ND	0.35±0.15 g	ND	ND

*Means with the same letter are not significantly different (LSD) according to ANOVA test ($p \leq 0.01$)

**ND = not detected, + = produced fluorescent pigment, - = not produced fluorescent pigment.

3.4 Characterization and Identification of Potential *Streptomyces*

3.4.1 Phenotypic characteristics

The growth of isolate RHI-39 and isolate RHI-146 was checked on various tested media, including yeast extract-malt extract (ISP-2), oatmeal (ISP-3), inorganic salt-starch (ISP-4), glycerol-asparagin (ISP-5), tyrosine (ISP-7) and Bennett's modified agar. On different agar, both isolates produced different colony colors and aerial spore masses depending on the substrate. The characteristics of isolate RHI-39 and isolate RHI-146 were closely related to the genus *Streptomyces*.

3.4.2 Cultural and physiological characterization, cell wall composition (DAP) and microscopic determination

Streptomyces isolates were checked for chemical analysis of DAP isomer in whole cell hydrolysates. It revealed that 79 isolates (52.7 %) were assigned to the group of *Streptomyces* as their whole cell hydrolysates were rich in *LL*-DAP and formed colonies characteristics of *Streptomyces*. Fifty-three isolates (35.3 %) contained *meso*-DAP which were classified as non-*Streptomyces*. Eighteen isolates (12 %) were classified as unidentified group. Cell wall peptidoglycans of isolate

RHI-39 and isolate RHI-146 contained *LL*-diaminopimelic acid, typical of cell wall chemotype I, as well as glutamic acid, alanine and glycine. Cell contained no diagnostic sugar components.

After grouping by colony color, eight main classes were observed and main colors were grey (34 %), brown (30 %), white (10 %), orange (10 %), yellow (8.6 %), green (3.3 %), black (2 %) and no-produced (2 %) (Table 2). On the basis of *in vitro* antagonistic activities against Fusarium wilt disease and sheath blight disease, we selected isolate RHI-39 and isolate RHI-146 for identification. Under light microscope and SEM, both substrate and aerial mycelia structure of isolate RHI-39 and isolate RHI-146 were observed. The substrate mycelium of isolate RHI-39 was brown and aerial mycelium was white. SEM observation, spores showed smooth surface and spore chains were straight spiral which contained generally 8-10 spores per chain. RHI-39 colonies appeared powdery in consistency with brown mycelium, produced brown pigment on ISP-2 medium which was classified as *Streptomyces* (Table 4). The substrate mycelium of isolate RHI-146 was yellow and aerial mycelium was white. Microscopic determination by SEM revealed that spores showed smooth surface and spore chains were straight spiral which contained generally 6-11 spores per chain (Figure 2). In addition, RHI-146 colonies appeared powdery in consistency with yellow mycelium, produced yellow pigment on ISP-2 medium and other media (Table 5).

Table 4. Morphological and biochemical characteristics of *Streptomyces* isolate RHI-39 on different culture media.

Culture media	*Streptomyces* isolate RHI-39			
	Growth rate	Aerial mycelium	Substrate mycelium	Pigment
ISP-1	Medium	Brown	Light orange	-
ISP-2	High	White	Orange	Brown
ISP-3	Medium	Brown	Brown	-
ISP-4	Medium	Brown	Yellow	-
ISP-5	High	White	Orange	-
ISP-6	Medium	Yellow	Light orange	-
ISP-7	High	White	Orange	-
ISP-8	Medium	Yellow	Light orange	-
ISP-9	Rare	Brown	Orange	-
Hickey-Tresner	Medium	Yellow	Brown	-
Nutrient agar	Medium	Yellow	Light orange	-
PDA	Medium	Light orange	Orange	-
Modified Bennett agar	Medium	Yellow	Brown	-

***** Color compared with Standard ISCC-NBS color chart [14]. *** *** ISP-1, Tryptone-yeast extract agar; ISP-2, Yeast extract-malt extract agar; ISP-3, Oatmeal agar; ISP-4, Inorganic salts-starch agar; ISP-5, Glycerol-asparagine agar; ISP- 6, Peptone-yeast extract-iron agar; ISP-7, Tyrosine agar; ISP-8, Nitrate medium; ISP-9, Carbon utilization agar

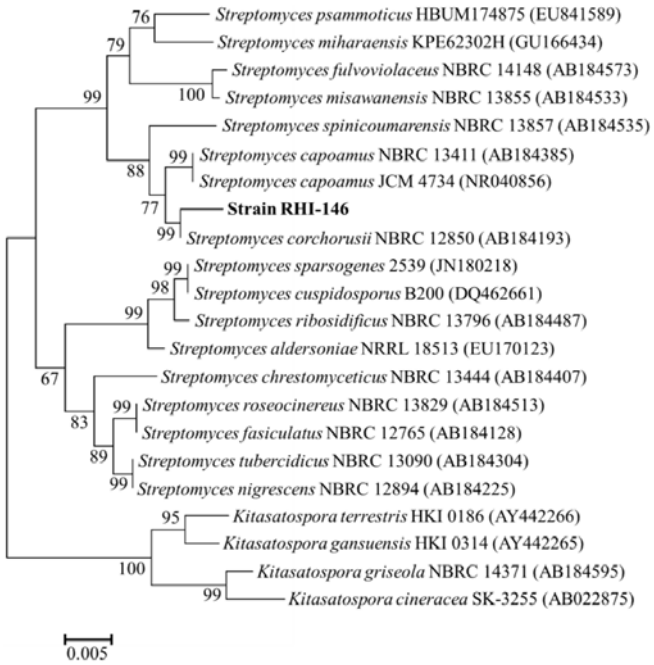

Figure 3. Neighbor-joining tree based on 16S rRNA gene sequences showing relationship among *Streptomyces* isolate RHI-146 and related *Streptomyces*. The numbers on branches indicated percentage bootstrap values of 1,000 replicates (only values > 50% are indicated). Bar represents 0.005 substitutions per nucleotide position.

Table 5. Morphological and biochemical characteristics of *Streptomyces* isolate RHI-146 on different culture media.

Culture media	*Streptomyces* isolate RHI-146			
	Growth rate	Aerial mycelium	Substrate mycelium	Pigment
ISP-1	Medium	Brown	Light orange	-
ISP-2	High	White	Yellow	Yellow
ISP-3	Medium	Brown	Light brown	-
ISP-4	Medium	Orange	Brown	-
ISP-5	High	Brown	Orange	-
ISP-6	Medium	Yellow	Light orange	-
ISP-7	High	Brown	Light orange	-
ISP-8	Medium	Yellow	Yellow	-
ISP-9	Rare	Brown	Brown	-
Hickey-Tresner	Medium	Brown	Orange	-
Nutrient agar	Medium	White	Light yellow	-
PDA	Medium	Light orange	Orange	-
Modified Bennett agar	high	Brown	Light orange	-

***** Color compared with Standard ISCC-NBS color chart [14] *** *** ISP-1, Tryptone-yeast extract agar; ISP-2, Yeast extract-malt extract agar; ISP-3, Oatmeal agar; ISP-4, Inorganic salts-starch agar; ISP-5, Glycerol-asparagine agar; ISP-6, Peptone-yeast extract-iron agar; ISP-7, Tyrosine agar; ISP-8, Nitrate medium; ISP-9, Carbon utilization agar

3.4.3 Molecular identification and phylogenic tree analysis

The 16S rDNA amplicons were sequenced and identified using the BLAST algorithm. The identified cultures belonged to the genus *Streptomyces*. BLAST search results indicated 16S rDNA sequence of RHI-39 isolate was closely related to *Streptomyces lydicus* with 90 % (1,267 bp) identity and RHI-146 isolate had close relationship to *Streptomyces corchorusii* with 99 % (1,349 bp) identity. Comparison of isolate RHI-39 and isolate RHI-146 nucleotide sequence with members of actinomycetes clearly showed that these isolates belonged to the genus *Streptomyces*. A neighbor-joining tree based on 16S rDNA sequences showed that the isolates occupied a distinct phylogenetic position with the radiation including representatives of the *Streptomyces* family. The phylogenetic tree based on Maximum-parsimony method also showed that the isolates formed a separate clade. Physicochemical parameters and 16S rDNA sequencing showed that RHI-146 isolates were identified as *Streptomyces corchorusii* (Figure 3).

3.5 Determination of Antifungal Activity of Potential *Streptomyces*

To determine antifungal activity produced by *Streptomyces*, crude extracts were prepared by solvent extraction and test for antifungal activities using agar well diffusion assay. Inhibition of mycelia growth was observed in the presence of crude extracts after incubation for 5 days. Crude extract of *Streptomyces* (14 isolates) inhibited mycelia growth of *Fusarium oxysporum* and *Rhizoctonia solani* AG-2. Crude extract of isolate RHI-146 exerted a high inhibitory activity over other crude extract samples (Table 6).

Table 6. Antifungal activity of culture filtrate and crude extract of *Streptomyces* against *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 using agar well diffusion method.

Isolates	*Fusarium oxysporum*		*Rhizoctonia solani* AG-2	
	Culture broth	Crude extract	Culture broth	Crude extract
RHI-11	13.3±1.5 def	13.7±2.1 de	22.3±1.2 c	23.3±1.5 bc
RHI-12	11.7±1.5 f	12.3±1.2 e	20.0±1.0 de	21.0±1.7 cd
RHI-27	12.7±1.2 ef	15.0±1.0 d	21.7±1.2 cd	23.3±0.6 bc
RHI-35	13.0±1.0 def	14.3±1.2 de	19.0±1.0 e	20.0±1.7 d
RHI-39	22.7±0.6 ab	24.7±0.6 a	22.3±0.6 c	24.0±1.0 b
RHI-55	15.3±0.6 d	15.7±1.5 d	23.0±1.0 bc	24.0±1.0 b
RHI-67	13.7±1.5 def	14.0±1.0 de	21.0±1.7 cd	23.0±1.7 bc
RHI-79	19.7±1.2 c	20.3±1.2 b	24.7±0.6 ab	26.7±0.6 a
RHI-81	13.7±1.2 def	12.3±0.6 e	21.3±0.6 cd	22.3±2.1 bc
RHI-99	20.0±1.0 c	19.3±1.5 bc	22.7±0.6 c	24.3±1.2 b
RHI-110	21.0±2.0 bc	19.0±1.0 bc	18.3±1.2 e	18.7±1.2 d
RHI-111	14.0±1.0 def	15.3±0.6 d	22.3±0.6 c	23.7±1.2 b
RHI-146	24.3±1.5 a	26.3±0.6 a	26.0±1.0 a	26.7±1.2 a
RHI-147	15.0±2.0 de	17.7±1.5 c	20.0±1.7 de	18.7±0.6 d

*Means with the same letter are not significantly different (LSD) according to ANOVA test ($p \leq 0.01$)

This research showed that *Streptomyces* sp. exhibited antifungal activities to *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 *in vitro*, which proved that rhizospheric soil of rice can serve as an effective source of biocontrol microorganisms.

4. DISCUSSION

Actinomycetes are abundantly distributed in the rhizosphere and colonized plant roots which play a role in plant growth promotion. In the present study, we isolated and characterized actinomycetes from rice rhizosphere samples. Rhizosperic soil was selected in this study because it contained twice as much *Streptomyces* isolates as non-rhizospheric soils [10]. Results indicated that *Streptomyces* sp. constituted the majority of total actinobacteria in the rice rhizosphere (10^6 cfu/g). The search for novel metabolites from *Streptomyces* sp. was done from rhizosphere of rice because *Streptomyces* have been found to have higher occurrences in rhizosphere soil than in bulk soil [18]. Plant exudates may play a role in the occurrence of microbial diversity as reported by Coombs and Franco (2003) [18] that actinobacteria were the abundant rhizosphere microflora and effective colonizers of plant root.

The use of actinobacteria to prevent plant pathogens and insect pests is supplementary for biological control of plant diseases. Phytopathogenic fungi are one of the major problems to agricultural production. *Streptomyces* sp. produced various bioactive compounds including antibiotics which used as agrochemicals [18]. Results indicate that 98 % of isolates were active against all tested fungi and percentage of active isolates varied by each color series. The most active compound producing isolates belong to grey and brown color series and 98 % were active against one of tested organisms. Most active *Streptomyces* were found in white and grey colony color series [18], however, some *Streptomyces* isolates (2 %) did not show any antibiotic activity (Table 2). It could be due to the possibility that these isolates produced other compounds which were not screened for activities in this study. Comparison of antifungal activity among all color classes against tested organisms showed that isolates in grey and brown series displayed highest antibiosis against fungal tested. Different percentage of antifungal activity could belong to different species of *Streptomyces* or different bioactive compounds. Current use of *Streptomyces* to control pathogenic fungi is little. *Streptomyces* isolate RHI-39 and *Streptomyces* isolate RHI-146 inhibited mycelium growth and spore germination of *Fusarium oxysporum* and *Rhizoctonia solani* AG-2. Our research agreed with *Streptomyces violaceusniger* G10 which showed antagonistic effects against *Fusarium oxysporum* f. sp. *cubense* and made hyphal swelling and inhibited spore germination of fungi [19]. Chitinase producing strain of *Streptomyces griseus* MG 3 possessed antifungal activity against *Aspergillus*, *Botrytis cinerea*, *Fusarium culmorum*, *Guignardia bidwelli* and *Sclerotia sclerotiorum* [20]. Also, *Streptomyces* strain CDRIL-312 and *S. purpueofuscu*s showed good antifungal activity against filamentous fungi [21]. However, there are limited reports of *Streptomyces* sp. which showed antagonistic effect to rice pathogens, i.e. *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 which caused yield losses up to 50 % [22]. The effects of *Streptomyces* sp. on fungal mycelia in dual culture assay were checked under SEM. They showed mycelia deformities near the zone of inhibition that were caused by antibiological compounds and extracellular enzymes (Figure 1). There are many reports related to antifungal substances which induce malfunctions such as stunting,

distortion, and swollen hypha protuberances of highly branched appearance of fungal germ tubes [22].

Biocontrol is one of the most desirable traits for *Streptomyces*. It has been reported that antifungal mechanism has been attributed to the action of hydrolytic enzymes such as chitinase, β-1,3-glucanase and protease [23]. Production of chitinase and β-1,3-glucanase by *Streptomyces* sp. was related to fungal growth inhibition and biocontrol of fungal pathogens due to the cell wall degrading enzyme activity [23]. Phytopathogenic fungi cell wall contains β-1,3-glucan, chitin and protein which are essential for disease transmission and pathogenesis. Cell lysis by β-1,3-glucanase/chitinase/protease-producing microbe leads to leakage of cell contents and collapse of fungal cell wall [24]. *Streptomyces* isolate RHI-16 produced the maximum chitinase (3.85 U/ml), followed by *Streptomyces* isolate RHI-40 (3.15 U/ml) and *Streptomyces* isolate RHI-42 (3.00 U/ml) (Table 3). Chitinase degraded fungal cell wall and limited growth of fungal pathogens. *Streptomyces* sp. produced various levels of chitinase. Rhizospheric *Streptomyces* with chitinolytic activity have been reported to significantly reduce the risk of pathogenic infection [24]. Bressan (2003) [25] reported the effectiveness of *Streptomyces* sp. isolated from maize rhizosphere that they inhibited seed pathogenic fungi, i.e. *Aspergillus* sp. and *Fusarium subglutinans* but did not suppress *Penicillium* sp. The culture filtrate of *Streptomyces* sp. inhibited mycelium growth of *Pythium utimum*, *Fusarium oxysporum* f. sp. *albedinis*, *Scherotium rolfsii*, *Verticillium dahlia* and *Botrylis cinerea* [26]. The non-significant chitinase producer, *Streptomyces* isolate RHI-39 and *Streptomyces* isolate RHI-146, inhibited all tested fungal pathogens indicating a chitinase-independent antifungal activity. This was consistent with results by Jog *et al.* (2014) [27].

Protease activity in culture filtrates of tested isolates was determined and results were presented in terms of enzyme units (Table 3). Isolate RHI-146 culture filtrate showed maximum protease activity (130 U/ml), followed by *Streptomyces exfoliates* CFS1068 (115.2 U/ml), and *Streptomyces somaliensis* GS 1242 (102.0 U/ml) [28]. Siderophore production of tested isolates were detected by color change on CAS agar and production of halo zone around colonies. Different color changes in CAS agar suggest the production of siderophores by a variety of microorganism isolated. Two major groups of bacterial siderophores, hydroxamate and catechol, at neutral pH are reddish-orange to yellow color respectively [29]. Siderophore is one of the compounds that stimulates plant growth by forming complex with iron (Fe^{3+}) in the rhizosphere, making iron unavailable for phytopathogens and developing growth of the plant. Our research, showed that 14.6 % of isolate were positive for siderophore production. Siderophore is usually produced by actinomycetes. It binds Fe^{3+} from the environment and makes Fe^{3+} available for microbial growth. Plants utilized siderophore-bound Fe^{3+} as an iron source. *Streptomyces* sp. isolated from rhizosphere soil produce siderophore and inhibit growth of phytopathogens. Rhizospheric *Streptomyces* sp. need to compete with other rhizosphere plant pathogens for iron, hence competition for iron is a possible mechanism to control the phytopathogens.

Streptomyces isolate RHI-39 and *Streptomyces* isolate RHI-146 inhibited mycelium growth and spore germination of *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 by various modes of action. Many studies refer *Streptomyces* isolates for plant protection from most phytopathogenic fungi.

They can survive in soils of various types, have long survival due to their spore forming ability, are capable of producing various bioactive compounds such as antibiotics, siderophores, chitinase, phytohormones and of phosphate solubilization [30]. Moreover, there are limited number of report of *Streptomyces* sp. that showed antagonistic ability to rice pathogens, i.e. *Fusarium oxysporum* and *Rhizoctonia solani* AG-2 which caused yield losses up to 50 %. Our study confirmed that *Streptomyces* are able to produce a wide variety of antifungal activity. The cultural and morphological characterization indicated that the isolate belongs to *Streptomyces* sp. A tree constructed by the Maximum-parsimony method showed that the isolate forms a distinct phyletic line from other related isolates available in the database (Figure 3). The isolate RHI-146 showed 99 % similarity with *Streptomyces corchorusii* NBRC 12850 on the culture data bank.

Data from the research proved that the highest antifungal activities not only displayed antagonistic activity characteristic but also displayed hydrolytic enzyme production. The potential for development of actinomycetes as a biocontrol agent was primarily indicated by the antagonistic activity mechanism including a). direct mechanism *via* an antibiosis or lysis mechanism, or b). indirect mechanism by inducing plant deference's and growth promoter substances. The antibiosis mechanism is facilitated by antibiotic compounds, and the lysis mechanisms are facilitated by amylase, protease, chitinase, cellulase and lipase produced by the actinomycetes. Additionally, indirect mechanism such as siderophore production and hydrogen cyanide characteristics combated disease development. Results showed that a combination of hydrolytic enzymes or other antagonistic mechanisms result in a higher level of antagonism than single mechanism alone (Table 1). Preliminary assessments of these characteristics are important for the best selection of biocontrol agent candidates. *Streptomyces* sp. showed higher antagonistic to rice fungal pathogen. The idea that ideal biological control agent suites native conditions is of the prime importance. *Streptomyces* sp. are especially significant for the following reasons: they can survive in soils of various types, have long survival due to their spore forming ability, are capable of producing various bioactive compounds, such as antibiotics, siderophores, chitinases, phytohormones and of phosphate solubilization [29]. Nowadays, biopesticides are applied using spray technologies, dipping or coating seed but the success is highly limited because of improper equipment, poor formation efficacy or combination of both [30]. The biocontrol agent which uses for leaf surface is exposed to rapidly fluctuating temperature and relative humidity. Leaf surface provides limited nutrient resources to bacterial colonies and most of phyllosphere bacterial colonies are easily killed by non-penetrating agents such as peroxide or UV light [31].

In conclusion, the research described the *in vitro* activity of actinomycetes which suppressed the growth of *Fusarium oxysporum* and *Rhizoctonia solani* AG-2, the causing agent of Fusarium wilt disease and sheath blight disease of rice *in vitro*, respectively. Preliminary analysis based on antifungal activity, hydrolytic enzyme and siderophore production facilitated the best selection of actinomycete candidates. Further researches are required to evaluate their potential as biocontrol under greenhouse and field conditions and to develop appropriate formulation to maintain the survival of biocontrol agent on the leaves and roots. *Streptomyces* sp. are likely to be the potential

candidates for discovery of novel secondary metabolites for various biocontrol application. Determination of the exact mechanisms of action of the biocontrol agents can assist the use of eco-friendly bio-fungicides in the future.

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REFERENCES

[1] Ou S.H., Rice diseases. Common wealth Mycological Institute, Kew, England, 1985: 380.

[2] Altinok H.H., *Plant Pathol. J*., 2005; **54**: 577. DOI 10.1111/j.1365-3059.2005.01235.x.

[3] Naidu V.D., *J. Res. Assam. Agric. Univ*., 1992; **10**: 78-80.

[4] Oard S., Rush M.C. and Oard J.H., *J. Appl. Microbiol*., 2004; **97**: 169-180. DOI 10.1111/j.1365-2672.2004.02291.x.

[5] Behal V., *Adv. Appl. Microbiol*., 2000; **47**: 113-156. PMID 12876796.

[6] Chamberlain K. and Crawford D.L., *J. Ind. Microbiol. Biotechnol*., 1999; **23**: 641-646. PMID 10455494.

[7] Hwang B.K., Lim S.W., Kim B.S., Lee J.Y. and Moon S.S., *Appl. Environ. Microbiol*., 2001; **67**: 3739-3745. DOI 10.1128/AEM.67.8.3739-3745.2001.

[8] Hwang B.C., Ahn S.J. and Moon S.S., *Can. J. Bot*., 1994; **72**: 480-485. DOI 10.1139/b94-064.

[9] Locci R., *Streptomycetes* and Related Genera; in Williams S.T., Sharpe M.E. and Holt J.G., eds., *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins, Baltimore, 1994; **4**: 2451-2508.

[10] Shirling E.B. and Gottlieb D., *Int. J. Syst. Bateriol*., 1966; **16**: 313-340. DOI 10.1099/00207713-16-3-313.

[11] Yung W.M. and Crawford D.L., *Appl. Environ. Microbiol*., 1995; **61**: 3119-3128. PMC 167587.

[12] Miller G.L., *Anal. Chem*., 1959; **31**: 426-428. DOI 10.1021/ac601479030.

[13] Singh P.P., Shin Y.C., Park C.S. and Chung Y.R., *Phytopathology*, 1999; **89**: 92-99. DOI 10.1094/PHYTO.1999.89.1.92.

[14] Goodfellow M., Kampfer P., Busse H.J., Trujillo M.E., Suzuki K., Ludwig W. and Whitemann W.B., *Bergey's Manual of Systematic Bacteriology*, 2nd Edn., vol. 5., Springer, New York, 2012.

[15] Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S., *Mol. Biol. Evol*., 2011; **28**: 2731-2739. DOI 10.1093/molbev/msr121.

[16] Ellaiah P., Adinarayana G., Saisha V. and Vasu P., *Indian J. Microbiol*., 2005; **45**: 33-36.

[17] Coombs J.T. and Franco C.M.M., *Appl. Environ. Microbiol*., 2003; **69**: 4260-4262. DOI 10.1128/AEM.69.7.4260-4262.2003.

[18] Getha K. and Vikineswary S., *J. Ind. Microbiol*., 2002; **28**: 303-310. DOI 10.1038/sj/jim/7000247.

[19] Hoster F., Schmitz J.E. and Daniel R., *Appl. Microbiol. Biotechnol*., 2005; **66**: 434-442. DOI 10.1007/s00253-004-1664-9.

[20] Harindran J., Gupte T.E. and Naik S.R., *World J. Microbiol. Biotechnol.*, 1999; **15**: 425-430. DOI 10.1023/A:1008934808486.

[21] Anupama M., Narayana K.J.P. and Vijayalakshimi M., *Res. Microbiol.*, 2007; **4**: 1-3.

[22] Soares A.C.F., Sousa C.S., Garrido M.S., Perez J.O. and Almeida N.S., *Braz. J. Microbiol.*, 2006; **37**: 456-461. DOI 10.1590/S1517-83822006000400010.

[23] Macagnan D., Romeiro R.D.A., Pomella A.M.V. and Desouza J.T., *Biol. Control*, 2008; **47**: 309-314. DOI 10.1016/j.biocontrol.2008.08.016.

[24] Bressan W., *Biol. Control.*, 2003; **48**: 233-240. DOI 10.1023/A: 1022673226324.

[25] Loqman S., Barka E.A., Clement C. and Ouhdouch Y., *World J. Microbiol. Biotechnol.*, 2009; **25**: 81-91. DOI 10.1007/s11274-008-9864-6.

[26] Jog R., Pandya M., Nareshkumar G. and Rajkumar S., *Microbiology*, 2014; **160**: 778-788. DOI 10.1099/mic.0.074146-0.

[27] Jain P.K., Jain R. and Jain P.C., *Hindustan Antibiot. Bull.*, 2003; **46**: 29-33. PMID 16281826.

[28] Milagres A.M.F., Machuca A. and Napaleao D., *J. Microbiol. Meth.*, 1999; **37**: 1-6. PMID 10395458.

[29] Hamdali H., Hafidi M., Virolle M.J. and Ouhdouch Y., *World J. Microbiol. Biotechnol.*, 2008; **24**: 2565-2575. DOI 10.1007/s11274-008-9817-0.

[30] Navon A., *Crop Protect.*, 2000; **19**: 669-676. DOI 10.1016/S0261-2194(00)00089-2.

[31] Lindow S.E. and Brandl M.T., *Appl. Environ. Microbiol.*, 2003; **69**: 1875-1883. DOI 10.1128/AEM.69.4.1875-1883.2003.