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ORIGINAL PAPER

Antagonistic potential of native strain *Streptomyces aurantiogriseus* VSMGT1014 against sheath blight of rice disease

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Abstract A total of 132 actinomycetes was isolated from different rice rhizosphere soils of Tamil Nadu, India, among which 57 showed antagonistic activity towards *Rhizoctonia solani*, which is sheath blight (ShB) pathogen of rice and other fungal pathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium udum* and *Alternaria alternata* with a variable zone of inhibition. Potential actinomycete strain VSMGT1014 was identified as *Streptomyces aurantiogriseus* VSMGT1014 based on the morphological, physiological, biochemical and 16S rRNA sequence analysis. The strain VSMGT1014 produced lytic enzymes, secondary metabolites, siderophore, volatile substance and indole acetic acid. Crude metabolites of VSMGT1014 showed activity against *R. solani* at 5 μg ml^{-1}; however, the prominent inhibition zone was observed from 40 to 100 μg ml^{-1}. Reduced lesion heights observed in culture, cells-free filtrate, crude metabolites and carbendazim on challenge with pathogen in the detached leaf assay. The high content screening test clearly indicated denucleation of *R. solani* at 5 μg ml^{-1} treatment of crude metabolite and carbendazim respectively. The results conclude that strain VSMGT1014 was found to be a potential candidate for the control of ShB of rice as a bio fungicide.

Keywords Antifungal · *Streptomyces aurantiogriseus* · *Rhizoctonia solani* · Sheath blight of rice · Detached leaf assay · High content screening

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Introduction

Sheath blight (ShB) of rice caused by *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris*) is a major disease of rice next to blast disease reduces both grain yield and quality (Lee and Rush 1983; Rush and Lee 1992). It is one of the most destructive diseases of rice that occurs globally (Slaton et al. 2003). The yield loss due to ShB was estimated between 6 and 50 % in other countries, whereas in India, it was recorded up to 69 % (Savary et al. 2000; Tang et al. 2007). Chemical fungicides are not very effective against soil borne diseases and they may damage the environment or cause human health problems. Microbial bio control agents have been promoted as an effective and environmentally friendly option to control the soil borne diseases. Among the microbes, actinomycetes are one of the most important groups of soil microorganisms which are significant producers of secondary metabolites such as antibiotics, pesticides, antiparasitic compounds, lytic enzymes (Omura 1992; Lange and Sanchez Lopez 1996; McCarthy and Williams 1990) and are being used as potential group of bio control agents.

Actinomycetes produce important therapeutically and agriculturally compounds (Tanaka and Omura 1993; Mincer et al. 2002). Soil actinobacteria are promising group of antifungal and root-colonizing microbes protect several plants

from soil borne fungal pathogens (Khaled El-Tarabily and Sivasithamparam 2006). Among actinomycetes, *Streptomyces* sp. is considered as predominant genus for producing more than 75 % of total bioactive molecules (Demain 2000). Actinomycetes play an important role in the rhizosphere by secreting a wide range of antimicrobial products, thus preventing the growth of common root pathogens. Apart from their bio control potential, the plant growth promoting traits were described (Gopalakrishnan et al. 2013; Nimaichand et al. 2013). *Streptomyces* are well known as antifungal agents against various plant pathogens (Errakhi et al. 2007; Li et al. 2011; Zacky and Tiny 2013; Cuppels et al. 2013). *Streptomyces* is known for their production of antimicrobial metabolites, hydrolytic enzymes, plant growth hormones, phosphate solubilization, hydrogen cyanide, siderophore and control plant diseases through local and systemic resistance (Conn et al. 2008; Harikrishnan and Shanmugaiah 2013). However, reports regarding the bearing of PGP traits, i.e., phosphate solubilization, organic acid production, siderophore production and secretion of numerous enzymes, which directly or indirectly help plant growth promotion, are scanty (Doumbou et al. 2001; Sadeghi et al. 2012). An effective and convenient method for the application of biocontrol agents against ShB of rice is a global necessity. *Jinggangmycin*, an antibiotic produced by *S. hygroscopicus* var. *jinggangensis*, is widely used for control of ShB of rice caused by *R. solani* in China (Shen 1996).

Indian soils are rich in microbial diversity, especially actinomycetes and the wealth of indigenous micro-flora of India has not been fully explored (Prabavathy et al. 2006). However, no work has been reported in India on the antifungal properties of *S. aurantiogriseus*. In our previous work (Harikrishnan and Shanmugaiah, 2013) we carried out a preliminary evaluation of selection and screening of actinomycetes against plant fungal pathogens. Based on the previous preliminary results we selected *Streptomyces* sp. VSMGT1014 to check the bio control mechanism. Hence, in the present work, the objective has been narrow down towards the controlling mechanism of *R. solani*, ShB pathogen. For this purpose, the efficiency of crude metabolites against ShB of rice through detached leaf assay and nuclear degradation of *R. solani* through high content screening system has been studied. Furthermore, we identified and studied the functional characteristics of potential strain *Streptomyces* sp. VSMGT1014.

Materials and methods

Isolation of soil borne actinomycetes

Rice rhizosphere samples were collected from rice fields in the Southern districts of Tamil Nadu, India and were stored at 10 °C until further use. Soil suspensions with 10 g soil were serially diluted and from each dilution, 0.1 ml aliquot was spread on International Streptomyces Project Medium-2 (ISP-2) (yeast extract—4, malt extract—10, dextrose—4, agar—20 [g l^{-1}]) with duplicates (Shirling and Gottlieb 1966) supplemented with 50 μg ml^{-1} of cyclohexamide, 50 μg ml^{-1} of nalidixic acid, plates were incubated at 28 °C for 7–10 days. Actinomycetes colonies on the agar plates were picked on the basis of their morphological characteristics and purified on ISP-2 agar.

Fungal strains and culture conditions

Five fungal plant pathogens such as *R. solani*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium udum* and *Alternaria alternata* were obtained from the Department of Microbial Technology, School of Biological Sciences, Madurai Kamaraj University. All the tested fungi were grown on potato dextrose agar (potato—200, glucose—20, agar—15 [g l^{-1}]), plates and incubated at 28 °C for 4–6 days. Stock cultures of pathogens were maintained on PDA slants and stored at 4 °C.

In vitro antagonism of *S. aurantiogriseus* VSMGT1014 against plant fungal pathogens

In vitro assessment of *S. aurantiogriseus* VSMGT1014 for antagonistic activity against *R. solani* was performed by the dual culture assay (Huang and Hoes 1976; Shanmugaiah et al. 2010) in PDA medium. All the isolates were screened for antagonism towards various fungal pathogens viz., *M. phaseolina*, *F. oxysporum*, *F. udum* and *A. alternata* by dual culture method. 5 days-old actinomycetes grown on ISP-2 agar were streaked in the periphery of each petri plate, 3 cm away from fungal disc and the plates were incubated at 28 °C up to 5 days.

Physiology and phenotypic characterization of *S. aurantiogriseus* VSMGT1014

The selected strain VSMGT1014 was identified based on their morphology characters, including spore morphology, substrate and aerial hyphae, pigment production and colony characteristics. The microscopic characterization was performed by cover slip culture method (Carrera et al. 2007). The mass color of mature sporulating aerial mycelium was observed following growth on ISP-2 plates. The spore morphology was observed under scanning electron microscope (SEM), the isolates was grown on the cover slides, air-dried in a desiccator and mounted on stubs, splutter-coated with gold and viewed at 11.74KX (FESEM, Supra 55, Carl Zeiss, Germany). All the biochemical tests were carried out according to Bergey's Manual of Determinative Bacteriology (Williams et al. 1994).

Genomic DNA extraction and molecular identification of *S. aurantiogriseus* VSMGT1014

The *S. aurantiogriseus* VSMGT1014 genomic DNA was extracted according to Hopwood et al. (1985). A 5 ml of VSMGT1014 culture was pelleted and suspended in 3 ml of TE buffer with 2 μg of lysozyme and incubated at 30 °C for 2 h. A 20 μl of EDTA (0.5 M), 100 μl of 10 % SDS and 100 μg of proteinase K was added, and then incubated at 37 °C for overnight. A 1 ml of Tris saturated phenol and 1 ml of chloroform was added with gentle mixing in between for 10 min. Centrifuged and the supernatant was washed with phenol:chloroform (1:1), followed by 1 ml of chloroform:isoamylalcohol (24:1) was added to the supernatant, mixed and centrifuged for 5 min at 4,000 rpm. RNase (1.5 μl of 12 μg ml^{-1} stock) treatment was given and incubated for 10 min. The supernatant was taken and 1 ml of chloroform:isoamylalcohol (24:1) was added, mixed and centrifuged for 5 min at 4,000 rpm. The upper layer was taken and 1/10 volume of 5 M NaCl and 4 volumes 100 % ethanol was added to DNA precipitation for 2 h and then washed with 70 % ethanol. The pellet resuspended with 1× TE buffer after drying. The integrity of DNA was checked by 0.8 % agarose gel electrophoresis. The DNA quality was evaluated in a Nanodrop 1000 (Thermoscientific).

The 16S rRNA was amplified using specific universal primers Actino F (5′-GGCCTTCGGGTTGTAAACC-3′) and Actino R (5′-CTTTGAGTTTTAGCCTTGCGGC-3′) (Kuske et al. 1998). PCR master mix (25 μl) contained 10 pmol of each primer, 20 ng of genomic DNA, 10× Taq DNA polymerase buffer (Sigma, USA), 2 U of Taq DNA polymerase, 10 mM of each dNTP, and 25 mM $MgCl_2$. The PCR reaction condition was as follows: 95 °C for 5 min, 35 cycles of 95 °C for 55 s, 55 °C for 45 s, 72 °C for 55 s and final extension at 72 °C for 10 min. Amplification was performed on a DNA thermo cycler (Smart PCR, Cyber Lab, USA) and electrophoresed on 1 % agarose gel. The PCR products were purified (PCR purification kit, Qiagen) and sequenced in an automated DNA sequencer with specific primers by a BigDye terminator cycle sequencing kit (PE Applied Biosystems, USA) on an ABI 310 automated DNA sequence (Applied Biosystems, USA). The 16S rRNA gene sequences were analyzed by the NCBI database using BLAST algorithms (Alschul et al. 1990). The evolutionary history was inferred by using the maximum likelihood method (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Biocontrol mechanism of *S. aurantiogriseus* VSMGT1014 against *R. solani*

The interaction of *S. aurantiogriseus* VSMGT1014 and *R. solani* was studied using a compound microscope (Labomed, USA) for the demonstration of antifungal activity. Both the pathogen and antagonist culture was co-inoculated on PDA and PDB up to 5 days. After 5 days, antagonist interaction zone mycelia in lacto phenol cotton blue (Himedia, India) was viewed under microscope at 40× to determine the occurrence of hyphal degradation. The *R. solani* mycelium alone was used as a control. Different bio control mechanisms have been studied using VSMGT1014 by standard techniques. Production of hydrogen cyanide (HCN) by VSMGT1014 was tested on nutrient sucrose agar medium (sucrose—5, yeast extract—4, peptone—4, beef extract—2, agar—18 [g l^{-1}]) supplemented with 4.4 % glycine (Lorck 1948) using sodium bicarbonate impregnated filter papers. A simple dual-bottom plate assay was performed to find out the effect of volatile compounds of *S. aurantiogriseus* VSMGT1014 on the mycelial growth and sclerotial formation in *R. solani*. Both culture and pathogen were inoculated on two different basal lids of PDA plates respectively. Both the basal lids were sealed with parafilm such that the *S. aurantiogriseus* VSMGT1014 inoculated lid was as upper and *R. solani* inoculated lid as the basal sides. An uninoculated PDA medium was kept for the control with *R. solani* in the basal lid. Plates were incubated at 28 °C and the growth of *R. solani* was observed after 3 days. Siderophore production by VSMGT1014 was determined (Schwyn and Neilands 1987). All antagonistic isolates were grown in King's B broth (King et al. 1954) for 48 h. The King's B broth medium amended with 2 μmol l^{-1} of filter sterilized $FeSO_4 \cdot 7H_2O$ served as control and *Pseudomonas fluorescens* CHAO strain was used as positive control. The cells were removed from the medium by centrifugation at 3,600*g* for 10 min and 1 ml of filter sterilized supernatant was mixed with 1 ml of the chrome-azurol S solution (Himedia, India). The change of the mixture color from blue to reddish brown within 15 min indicated the presence of siderophores. For the production of phosphate solubilization, the fresh culture of *S. aurantiogriseus* VSMGT1014 were spotted inoculated on Pikovskaya medium (Pikovskaya 1948) (pH 6.8) and incubated at 28 °C for 5 days for observing a clear zone around the colonies. Qualitative assay was performed to determine the production of lytic enzymes such as chitinase, cellulase, gelatinase, protease, amylase and pectinase production by VSMGT1014 using nutrient agar supplemented with 1 % of respective substrates.

Production of indole acetic acid

The production of indole acetic acid (IAA), by *S. aurantiogriseus* VSMGT1014 was determined using the method of Gordon and Weber (1951). A 100 μl of VSMGT1014 culture was inoculated into 100 ml of yeast malt extract broth containing 0.2 % L-tryptophan and incubated at

28 °C on a rotary shaker at 125 rpm for 7 days. Cultures were harvested and centrifuged at 11,000*g* for 15 min at 4 °C. The reaction mixture, included 1 ml of supernatant and 2 ml of the Salkowski reagent, was incubated at 25 °C for 30 min in the dark. Development of pink color indicates the production of IAA. The reaction was measured at 530 nm, and the IAA production (mg ml^{-1}) of the culture filtrate was quantified using a standard curve with known concentration of pure indole-3-acetic acid (Sigma, USA).

Extraction and characterization of antifungal metabolites

The *S. aurantiogriseus* VSMGT1014 was cultured in 50 ml flasks of the ISP-2 broth medium at 37 °C on a rotary shaker at 150 rpm for 4 days. A 1 ml of fresh culture (inoculum) was transferred into 1,000 ml Erlenmeyer flask containing 250 ml of ISP-2 broth and grown on a rotary shaker at 150 rpm for 6 days. The culture broth (1 l) was centrifuged at 11,000*g* for 15 min at 4 °C. Antifungal compounds were purified from the filtrate by solvent extraction method (Shanmugaiah et al. 2010). Cells free culture filtrate was mixed with ethyl acetate in the ratio of 1:1 (v/v) and was extracted manually by shaking vigorously for 1 h. The ethyl acetate soluble organic fraction was concentrated by a rotary evaporator (Buchi- R- 210/R-215) to obtain the crude extract. Thin layer chromatography (TLC) of the crude compounds was eluted with petroleum ether:ethyl acetate (4:1) solvent system in a pre-coated silica gel TLC plates of grade F274 (E-Merck, Germany). The eluted plates were dried completely and visualized under UV and iodine subsequently; the movement of the crude compound along with solvent was measured (Rf value). The crude metabolite was observed in UV/VIS spectrophotometer (Shimadzu 1800) in the UV range (200–400 nm) and FTIR (Shimadzu 8400S) spectrum were scanned in the 400–4,000 cm^{-1} range and plotted as intensity versus a wave number. The ethyl acetate extracts were again dissolved in 50 % methanol, centrifuged at a 10,000*g* prior to injection (10 µl) into an LCQ Fleet mass spectrometer coupled with HPLC (Thermo Fisher, MA, USA). Compounds were separated on a Prodigy C18 (5 µm, 50 × 4.60 mm) column (Phenomenex) using the following gradient (solvent A: 50 % methanol in water, solvent B: water, flow rate 0.2 ml min^{-1}). A mass spectrum was acquired in positive ion mode with the capillary voltage set to 1.3 kV. Detection was by ultraviolet and by positive electrospray MS.

Effect of crude extracts on fungal mycelial growth

The concentrated crude extract was dissolved in ethyl acetate and the crude extract was added into each well from 5 to 100 µg ml^{-1} made on PDA plate. Mycelial discs (9 mm in diameter) of 5 days old fungal pathogens (*R. solani*, *M. phaseolina*, *F. oxysporum*, *F. udum* and *A. alternata*) were placed on the center of the PDA plates, and then the plates were incubated at 28 °C. Plates were prepared in triplicate for each treatment. The mycelial growth of fungus was recorded after 3 days.

Detached leaf bio assay

The culture (cells-free culture filtrates and crude metabolites) of *S. aurantiogriseus* VSMGT1014 and carbendazim were tested against ShB of rice by the method of detached leaf assay (Guleria et al. 2007). Liquid culture of *S. aurantiogriseus* VSMGT1014 (previously grown as described above) was prepared to have 10^8 CFU ml^{-1}. In addition, culture filtrates of VSMGT1014 from 6 days old culture grown on ISP-2 broth was diluted with sterile water to a final concentration of 10 % (v/v). The crude metabolite (50 µg ml^{-1}) was prepared as described above. Leaf segments from 40 days old seedlings were taken from rice fields and brought to the laboratory in an ice box. The leaves were cropped in uniform size of 10 cm and placed in sterilized glass Petri dishes containing moisturized filter paper. Rice leaves were surface sterilized with 2 % sodium hypochlorite for 1 min, washed with distilled water and placed in a closed glass chamber (Badosa et al. 2009). Cell suspensions (10 % of 10^8 CFU) and cells-free culture filtrate of VSMGT1014 at 50 µg ml^{-1} crude extracts and 50 µg ml^{-1} carbendazim were sprayed on the surface of sterilized leaves in the Petri dish respectively. Sterilized glass slides (surface sterilized with absolute ethanol) were placed on the edges of leaves to maintain flatness. In each treatment, the abaxial surface of detached leaves was lightly wounded by pricking gently with a sterilized needle and *R. solani* agar plug was inoculated to the wound site (Song et al. 1993; Zheng et al. 2010) at the center of each leaf. Leaves sprayed with sterile distilled water were inoculated with *R. solani* agar plug to serve as control. The trays were incubated in a growth chamber at 25 ± 20 °C and 16 h light. After 2, 3 and 5 days of incubation, the lesion areas around each wound were assessed for ShB disease incidence and compared with control leaves. Three replicates were maintained for all the treatments. The lesion length along the leaves was measured and ShB severity was rated by the relative lesion height (RLH) method (Sharma et al. 1990) with the following formula.

$$\%\,\mathrm{RLH} = 100 \times \text{Total height of lesions}/\text{Total leaf height}$$

Effects of crude extracts of *R. solani* mycelial morphology

Different 250 ml flasks of *R. solani* growing in potato dextrose broth (PDB) were inoculated with 5 µg ml^{-1} crude extract and carbendazim and allowed to grow for

3 days on a rotary shaker at 120 rpm. After 3 days *R. solani* was fixed in 4 % formalin. A sample of mycelia (100 µl) was placed on a microscopic slide, squashed with a cover slip and transferred into 96 costar flat bottom plate. The mycelia was stained with Hoechst 33258 (5 µl ml^{-1}) for 15 min at room temperature in the dark and later visualized in High content screening system (Perkin Elmer-Operetta) using Xenon Fiber optic light source (Perkin Elmer Cermax). Sixteen fields were analyzed for the each treatment for mycelial morphology and number of nuclei.

Statistical analysis

The data were analyzed by analysis of variance and the treatment means were compared through least significant difference (LSD) value of duncans multiple range test (DMRT) test at $P < 0.05$ using Costat statistical software (Cohort Berkeley, CA, USA).

Results

Characterization and identification of actinomycete isolates

Among 132 isolates screened under a dual plate assay, 57 showed inhibitory activities against *R. solani*. The zone of inhibition towards *R. solani* ranged from 5 to 30 mm (Table 1). Isolate VSMGT1014 showed the largest zone of inhibition against the *R. solani*; and it was therefore selected for further studies. Dual plate assays of all the isolates against other fungal pathogens *M. phaseolina*, *F. oxysporum*, *F. udum* and *A. alternata* gave varying sizes of inhibition zones (Table 1). Characterization of isolate VSMGT1014 based on the morphological, physiological, biochemical and scanning electron microscopic observations identified it as *Streptomyces* sp. (Fig. 1). The aerial mycelium mass of isolate VSMGT1014 was gray in color with flexuous spore chains. Spores were oval shaped and had a smooth surface. The 16S rRNA sequence of strain VSMGT1014 showed high similarity (97–100 %) to *S. aurantiogriseus*, therefore it was designated as *S. aurantiogriseus* VSMGT1014. The 16S rRNA sequence analysis showed 100 % homology and close to *S. aurantiogriseus* strain 15747. The 16S rRNA sequence of strain VSMGT1014 was deposited in GenBank with accession number JX173962.

Biocontrol mechanism of *S. aurantiogriseus* VSMGT1014 against *R. solani.*

The *R. solani* mycelia were obtained from the periphery of the inhibition zone of a 5 days old dual culture. Observations under the microscope showed that the mycelia from dual cultures was deformed, disintegrating and failed to form fruiting bodies (sclerotium) compared to the control where *R. solani* was grown alone (Fig. 2).

Production of secondary metabolites

The 57 isolates that were observed to have inhibitory effects against *R. solani* were tested for production of plant growth promoting metabolites. Twenty three out of the 57 isolates tested were siderophore producers, 40 isolates produced IAA and 6 isolates were found to have phosphate solubilization abilities when compared with control (Table 2). Production of volatile metabolites assessed in dual-bottom plate assay revealed that among the 57 antagonistic isolates, 34 produced volatile substances. In addition, development of sclerotium by *R. solani* was completely arrested by volatile substances. Characterization of the antagonistic isolates for production of different enzymes revealed that among the 57, 22 isolates produced pectinase, while 17, 15, 11, 7 and 5 isolates produced amylase, gelatinase, protease, chitinase and cellulase respectively. None of these isolates, including VSMGT1014 produced HCN. *Streptomyces aurantiogriseus* had been reported for its antifungal action against phytopathogenic fungi (Phay et al. 1996) and algicidal action against cyanobacterium *Microcystis aeruginosa* (Somdee et al. 2013).

Characterization of antifungal metabolites

Five different compounds were visualized on TLC from crude metabolite with Rf values such as 0.46, 0.51, 0.61, 0.85 and 0.89. The UV spectrum of crude extracts showed λ max at 227–235 nm. FTIR spectrum of ethyl acetate extracts exhibited absorption at 2,956.97 and 2,924.18 cm^{-1}, which indicated acidic hydroxyl groups and the absorption in 1,649.19 cm^{-1} indicating a double bond of amide. Also absorption observed at 3,367.82 cm^{-1}, which indicated alcoholic hydroxyl group stretching. Absorption at 1,406.15 cm^{-1} indicates phenolic compounds, whereas absorption at 1,649.19, 1,020.38 and 1,074.39 cm^{-1} indicates the presence of primary amines. Another one absorption at 2,854.74 cm^{-1} indicated organic compounds having alkyl CH's. LC–MS analysis of ethyl acetate 14 extracts revealed molecular ions (*m/z* 369.35 and 418.36) and showed a characteristic absorption band in its UV spectrum, with absorbance maxima at 254 nm.

Effect of crude extract of *S. aurantiogriseus* VSMGT1014 on fungal pathogens

The zone of inhibition observed in plates inoculated with fungal pathogens increased with increasing concentration of crude extracts (Table 3). However, *R. solani, M.*

Table 1 Antifungal activity of actinomycete isolates against fungal pathogens

Isolates	Zone of inhibition (ZOI) in mm				
	R. solani	*M. phaseolina*	*F. oxysporum*	*F. udum*	*A. alternata*
VSMGT1013	14.0 ± 1	10.7 ± 1.5	5.5 ± 0.5	5.1 ± 1.1	8.0 ± 0.5
VSMGT1014	30.0 ± 0.2	20.5 ± 0.5	16.8 ± 0.8	18.8 ± 0.3	20.2 ± 0.7
VSMGT1015	19.5 ± 0.4	15.8 ± 0.1	12.1 ± 0.8	12.5 ± 0.5	15.0 ± 0.7
VSMGT1020	19.3 ± 0.6	15.5 ± 0.6	10.0 ± 0.9	17 ± 0.7	12 ± 0.5
VSMGT1021	20.0 ± 0.7	20.2 ± 0.6	12.5 ± 0.5	17 ± 0.8	19.5 ± 0.6
VSMGT1022	20.2 ± 0.3	10.6 ± 0.4	12.0 ± 0.3	14.3 ± 0.5	14.5 ± 0.3
VSMGT1023	23.0 ± 0.5	22.0 ± 0.5	6.5 ± 0.3	7.0 ± 0.6	18.5 ± 0.3
VSMGT1028	12.5 ± 0.4	18.8 ± 0.4	10.0 ± 0.7	11.5 ± 0.7	15.5 ± 0.7
VSMGT1029	14.5 ± 0.9	14.8 ± 0.9	10.8 ± 0.3	12.6 ± 0.6	13.0 ± 0.6
VSMGT1030	17.8 ± 1	10.8 ± 0.4	10.5 ± 0.8	14.0 ± 0.4	19.0 ± 0.6
VSMGT1031	21.8 ± 1.4	15.8 ± 1.1	17.5 ± 1.2	13.5 ± 1.3	19.8 ± 1.1
VSMGT1032	20.8 ± 0.1	10.8 ± 0.8	12.8 ± 0.8	12.0 ± 1.2	14.3 ± 0.5
VSMGT1033	21.8 ± 0.6	25.0 ± 1.3	11.8 ± 0.1	12.5 ± 0.2	16.5 ± 0.7
VSMGT1035	22.0 ± 0.5	18.8 ± 1.2	15.8 ± 1.8	12.8 ± 1.5	14.3 ± 1.5
VSMGT1036	19.0 ± 1.3	20.3 ± 1.6	22.3 ± 0.5	10.5 ± 1.3	14.8 ± 0.9
VSMGT1042	9.0 ± 0.9	10.0 ± 1.1	10.5 ± 1.4	12.8 ± 0.5	13.5 ± 1.4
VSMGT1043	6.8 ± 0.9	6.0 ± 0.6	5.0 ± 1.3	6.9 ± 0.5	10.0 ± 1
VSMGT1045	21.5 ± 1.5	18.8 ± 1	10.8 ± 0.6	12.8 ± 0.8	12.0 ± 1.9
VSMGT1046	22.8 ± 1.5	19.0 ± 1.1	14.0 ± 1.2	12.9 ± 1.2	18.0 ± 0.5
VSMGT1048	5.4 ± 0.5	10.0 ± 1	11.8 ± 0.1	10.0 ± 1.2	9.0 ± 0.5
VSMGT1051	5.5 ± 0.9	9.5 ± 0.4	10.6 ± 0.4	12.7 ± 0.4	12.0 ± 1
VSMGT1054	6.0 ± 1	7.0 ± 1.2	10.2 ± 0.1	10.8 ± 0.2	12.6 ± 0.3
VSMGT1055	13.8 ± 0.2	10.6 ± 1	10.0 ± 0.9	13.6 ± 0.8	15.0 ± 1.1
VSMGT1056	7.5 ± 0.4	8.0 ± 0.7	8.5 ± 1	10.7 ± 0.4	10.5 ± 0.4
VSMGT1057	13.0 ± 1.5	20.0 ± 1.5	14.8 ± 0.5	12.5 ± 0.7	15.7 ± 0.6
VSMGT1059	23.8 ± 0.7	19.5 ± 0.7	15.9 ± 0.7	16.8 ± 0.5	16.5 ± 0.2
VSMGT1060	22.0 ± 1.3	20.0 ± 0.5	6.5 ± 0.2	9.0 ± 0.9	10.8 ± 0.9
VSMGT1062	6.5 ± 0.2	9.0 ± 0.6	6.0 ± 0.9	9.0 ± 0.7	9.0 ± 1
VSMGT1063	18.3 ± 0.6	19.6 ± 0.1	10.9 ± 0.9	6.0 ± 0.4	10.4 ± 0.4
VSMGT1064	9.8 ± 0.9	10.5 ± 0.4	10.8 ± 0.6	10.0 ± 0.3	10.2 ± 1.1
VSMGT1066	11.5 ± 0.4	14.3 ± 0.6	15.4 ± 0.5	10.9 ± 0.9	14.0 ± 0.4
VSMGT1067	18.5 ± 0.4	18.0 ± 0.5	16.4 ± 0.4	14.8 ± 0.6	15.0 ± 0.7
VSMGT1068	18.8 ± 0.6	18.2 ± 0.9	14.9 ± 0.4	8.0 ± 1.2	12.0 ± 0.8
VSMGT1069	5.0 ± 0.6	8.5 ± 0.5	10.8 ± 1	9.5 ± 0.5	10.0 ± 0.8
VSMGT1070	5.3 ± 0.7	10.0 ± 1.0	13.7 ± 0.4	10.6 ± 0.3	10.8 ± 0.3
VSMGT1071	26.0 ± 1.5	19.8 ± 0.5	15.9 ± 0.5	14.5 ± 1.0	16.0 ± 0.7
VSMGT1072	24.0 ± 0.2	15.0 ± 0.8	16.0 ± 0.8	14.0 ± 0.3	10.0 ± 0.6
VSMGT2001	21.3 ± 0.4	18.6 ± 0.3	8.5 ± 0.5	8.3 ± 0.7	8.0 ± 0.5
VSMGT2002	19.5 ± 0.4	20.0 ± 0.7	14.5 ± 1.0	15.0 ± 0.9	13.0 ± 0.6
VSMGT4002	19.0 ± 1.1	19.5 ± 0.7	12.0 ± 0.9	14.8 ± 0.2	13.5 ± 0.6
VSMGT4004	12.0 ± 0.8	16.5 ± 0.6	15.0 ± 0.7	10.0 ± 0.7	12.0 ± 0.9
VSMGT4008	15.0 ± 0.3	17.8 ± 1.1	12.8 ± 0.1	12.0 ± 0.4	10.5 ± 0.2
VSMGT4012	23.0 ± 0.9	16.5 ± 0.3	15.5 ± 0.1	12.5 ± 0.3	10.8 ± 0.1
VSMGT4015	17.0 ± 0.5	18.6 ± 0.2	12.5 ± 0.5	10.8 ± 0.6	10.5 ± 0.4
VSMGT4016	19.0 ± 0.4	15.0 ± 0.5	10.2 ± 1.1	12.6 ± 0.4	12.5 ± 0.6
VSMGT4017	19.0 ± 0.7	20.0 ± 1	14.5 ± 0.4	13.5 ± 0.7	10.7 ± 1.2
VSMGT4024	17.0 ± 0.7	18.5 ± 1	12.8 ± 0.8	15.0 ± 0.5	15.5 ± 0.9
VSMGT5004	28.0 ± 1.3	20.0 ± 1.1	15.0 ± 0.6	12.6 ± 0.9	7.3 ± 0.9

Table 1 continued

Isolates	Zone of inhibition (ZOI) in mm				
	R. solani	*M. phaseolina*	*F. oxysporum*	*F. udum*	*A. alternata*
VSMGT5006	5.0 ± 0.9	8.0 ± 1.2	5.0 ± 0.4	5.0 ± 0.5	5.3 ± 0.4
VSMGT5007	26.0 ± 0.9	15.0 ± 0.8	15.5 ± 0.2	10.0 ± 0.5	13.6 ± 0.5
VSMGT5011	6.0 ± 0.7	10.5 ± 0.5	10.0 ± 0.6	12.5 ± 0.7	10.8 ± 0.7
VSMGT5016	19.0 ± 0.4	15.0 ± 0.7	9.0 ± 1.1	10.7 ± 0.4	13.5 ± 1.0
VSMGT5020	26.0 ± 1.5	20.3 ± 0.7	10.5 ± 0.6	9.0 ± 0.8	7.0 ± 0.9
VSMGT5021	12.0 ± 1.1	15.6 ± 0.7	13.6 ± 0.7	14.5 ± 1	10.0 ± 1.0
VSMGT5024	21.5 ± 0.7	19.8 ± 0.5	12.0 ± 1.3	8.0 ± 0.7	10.5 ± 0.8
VSMGT5025	23.0 ± 0.9	18.0 ± 0.8	14.0 ± 0.7	8.0 ± 1.0	11.3 ± 0.7
VSMGT5026	25.0 ± 0.6	13.0 ± 0.4	7.0 ± 0.5	10.5 ± 1.1	10.8 ± 1.1

Values are mean of three replications; ± standard deviation

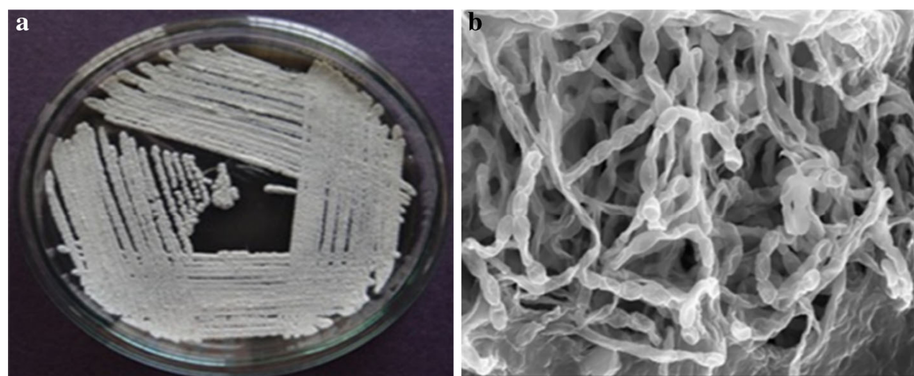

Fig. 1 **a** Morphology and scanning electron micrograph of *S. aurantiogriseus* VSMGT1014 grown on ISP-2 agar. **b** SEM-magnification = 11.74KX, WD = 11.6 mm, EHT = 20.00 kV

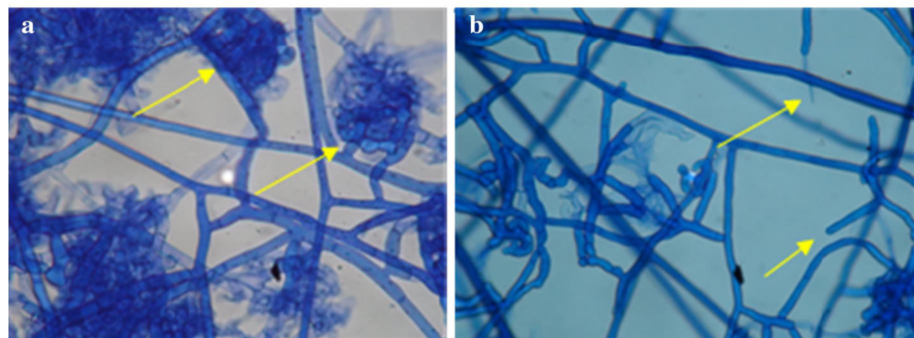

Fig. 2 Interaction between *R. solani* and *S. aurantiogriseus* VSMGT1014 under light microscope (magnification-40×): **a** *R. solani* alone, **b** *R. solani* + VSMGT1014 culture. *Arrow* indicates in (**a**)—hyphal and fruiting body; in (**b**)—hyphal and fruiting body (sclerotium) disintegration

phaseolina and *F. oxysporum* displayed larger zones of inhibition than *A. alternata* and *F. udum*. Only treatments with crude extract concentrations between 90 and 100 µg ml^{-1} showed significant inhibition. In Petri dishes inoculated with *A. alternata* and *F. udum* treatment with concentrations above 60 µg ml^{-1} did not significantly increase the inhibition zone. In the case of *M. phaseolina* treatment with crude extract concentrations above 40 µg ml^{-1} did not significantly increase the zone of inhibition. Mycelial growth of all the pathogens was inhibited by concentrations as low as 5 µg ml^{-1}. In addition, *R. solani* and *F. udum* displayed the smallest zones of inhibition when treated with low concentrations of the crude extract, but *R. solani* and *F. udum* showed the largest and smallest zones of inhibition respectively, when treated with the highest concentration of the extract compared to the other fungal pathogens (Table 3). Inhibition of growth was varied with each pathogen.

Table 2 Hydrolytic enzyme production, IAA production, siderophore, HCN and phosphate solubility of actinomycete isolates

Designation	Lytic enzyme production[a]						IAA[b]	Phosphate solubilization[c] (halo ≥ 5 mm)	Siderophore[c]	HCN[c]	Volatile[d]
	Chitinase	Cellulase	Gelatinase	Protease	Amylase	Pectinase					
VSMGT1013	−	−	−	−	−	−	++	−	−	−	−
VSMGT1014	+	+	+	+	+	+	+++	−	+	−	++
VSMGT1015	−	+	+	+	+	−	+	+	−	−	−
VSMGT1020	−	−	−	−	+	−	+	−	+	−	−
VSMGT1021	−	−	+	−	+	+	++	−	−	−	+
VSMGT1022	−	−	−	−	+	++	++	−	−	−	−
VSMGT1023	−	−	−	−	+	+	+++	−	+	−	+++
VSMGT1028	−	−	−	−	++	++	++	−	+	−	+
VSMGT1029	−	−	−	−	−	−	−	−	−	−	−
VSMGT1030	−	−	−	−	+	+	++	−	−	−	−
VSMGT1031	−	−	−	−	−	−	+++	−	−	−	+
VSMGT1032	−	−	−	−	−	−	−	−	−	−	+
VSMGT1033	−	−	−	−	+	++	+++	−	−	−	+
VSMGT1035	−	−	−	−	+	++	++	−	−	−	−
VSMGT1036	−	−	+	−	−	−	+	−	−	−	−
VSMGT1042	−	−	−	−	−	+	+	−	+	−	−
VSMGT1043	−	−	−	−	−	−	+++	−	−	−	−
VSMGT1045	−	−	−	−	+	−	+	+	+	−	+
VSMGT1046	−	−	−	−	−	−	+++	−	−	−	−
VSMGT1048	−	−	−	++	−	−	−	−	−	−	+
VSMGT1051	+	−	−	+++	−	−	++	−	−	−	+
VSMGT1054	−	−	−	−	−	+	+++	−	+	−	+
VSMGT1055	−	−	−	−	−	−	−	−	+	−	−
VSMGT1056	−	−	−	−	−	−	++	−	+	−	+
VSMGT1057	+	−	−	−	+	+++	+	−	−	−	+
VSMGT1059	−	+	−	−	−	−	−	−	+	−	+
VSMGT1060	−	−	−	−	−	+	−	−	−	−	−
VSMGT1062	−	+	+	+++	−	−	−	−	−	−	+
VSMGT1063	−	−	−	+	−	+	−	−	−	−	−
VSMGT1064	−	−	−	−	−	−	++	−	−	−	++
VSMGT1066	−	−	−	−	−	−	++	−	−	−	+
VSMGT1067	−	−	−	−	−	+	−	−	−	−	++
VSMGT1068	−	−	−	−	−	−	++	−	−	−	+
VSMGT1069	−	−	+	−	−	−	+	−	+	−	−

Table 2 continued

Designation	Lytic enzyme production[a]						IAA[b]	Phosphate solubilization[c] (halo ≥ 5 mm)	Siderophore[c]	HCN[c]	Volatile[d]
	Chitinase	Cellulase	Gelatinase	Protease	Amylase	Pectinase					
VSMGT1070	−	−	+	−	−	+++	+	−	+	−	−
VSMGT1071	−	−	−	−	−	−	−	−	+	−	++
VSMGT1072	−	−	−	−	−	+	−	−	−	−	++
VSMGT2001	−	−	−	−	+	−	−	−	−	−	++
VSMGT2002	−	−	+	−	−	−	++	−	+	−	+
VSMGT4002	−	−	−	−	−	−	−	−	+	−	+
VSMGT4004	−	−	−	−	−	−	++	−	−	−	−
VSMGT4008	−	−	−	−	−	−	++	−	−	−	+
VSMGT4012	−	−	++	+	−	++	++	−	+	−	−
VSMGT4015	−	−	+	−	+	++	+	−	+	−	+
VSMGT4016	+	−	+	−	−	++	++	−	+	−	+
VSMGT4017	+	−	+	++	+	++	−	+	−	−	−
VSMGT4024	−	−	−	++	−	−	+++	−	−	−	−
VSMGT5004	−	−	−	−	−	−	++	−	−	−	+
VSMGT5006	−	+	+	−	−	−	++	−	+	−	+
VSMGT5007	+	−	+++	+++	+	+	+++	−	+	−	++
VSMGT5011	−	−	−	−	−	−	+++	−	+	−	+
VSMGT5016	+	−	+++	+++	−	−	++	++	+	−	+
VSMGT5020	−	−	−	−	−	−	−	−	+	−	−
VSMGT5021	−	−	−	−	−	−	−	−	+	−	+
VSMGT5024	−	−	−	−	−	+	+++	+	−	−	++
VSMGT5025	−	−	−	−	−	−	++	+	−	−	+
VSMGT5026	−	−	−	+	+	−	−	−	−	−	−

[a] For hydrolytic enzymes, +++ >15 mm, ++ >10 mm, + >5 mm, −− <5

[b] For IAA, +++ >2, ++ >1, + >0.5, −− <0.5

[c] For Siderophore, HCN and phosphate solubility, + positive, − negative

[d] For volatile activity. +++ >6, ++ 4–6 mm, + 2–4 mm, −− <2 mm

Table 3 Effect of crude metabolites of VSMGT1014 on fungal pathogens under in vitro conditions

Concentration (μg ml^{-1})	Zone of inhibition(mm)				
	R. solani	*A. alternata*	*M. phaseolina*	*F. udum*	*F. oxysporum*
5	6.0^{h}	12.25^{d}	14.5^{e}	8.25^{d}	13.25^{h}
10	8.5^{g}	12.75^{d}	16.0^{d}	9.5^{cd}	14.5^{gh}
20	12.5^{f}	12.5^{d}	18.0^{c}	9.5^{cd}	15.25^{fg}
30	13.75^{f}	12.75^{d}	19.5^{b}	9.75^{c}	15.75^{efg}
40	14.0^{ef}	13.0^{cd}	20.5^{ab}	10.5^{bc}	16.5^{def}
50	15.5^{e}	13.5^{bcd}	20.25^{ab}	10.75^{bc}	16.75^{de}
60	20.0^{d}	14.25^{abc}	20.25^{ab}	11.5^{ab}	17.5^{cd}
70	22.0^{c}	14.25^{abc}	20.75^{ab}	11.75^{ab}	17.5^{cd}
80	23.5^{bc}	14.5^{ab}	21.0^{ab}	12.25^{a}	18.5^{bc}
90	24.5^{b}	14.75^{ab}	21.5^{a}	12.5^{a}	19.5^{ab}
100	26.5^{a}	15.5^{a}	21.75^{a}	12.75^{a}	20.5^{a}
LSD ($P = 0.01$)	1.53	1.19	1.39	1.23	1.26

Means followed by same letter did not differ significantly by Duncans multiple range test (DMRT) of ANOVA (analysis of variance); $P = 0.01$ significance level; means are average of four replications. Control for each pathogen was applied with sterile water and treated as no zone of inhibition

Table 4 Control of sheath blight of rice by the crude metabolites of *S. aurantiogriseus* VSMGT1014

Treatment	% Relative lesion height after 5 days
R. solani alone	100.0^{a}
R. solani + culture (10^{8} cfu/ml)	76.33^{bc}
R. solani + culture filtrate	78.0^{b}
R. solani + crude extract	72.0^{c}
R. solani + carbendazim	73.0^{c}
LSD ($P = 0.01$)	4.53

Means followed by same letter did not differ significantly by Duncans multiple range test (DMRT) of ANOVA (analysis of variance); $P = 0.01$ significance level; means are average of three replications

Effect of *S. aurantiogriseus* VSMGT1014 against sheath blight of rice

Treatment detached rice leaves with either cell culture, culture filtrate, or crude extract significantly reduce the percent lesion height compared to the control (Table 4). Reduction in percent lesion height was not significantly different between leaves treated with the culture of *S. aurantiogriseus* VSMGT1014, crude extract and the fungicide carbendazim after 5 days of incubation. However the crude extract and carbendazim gave the highest reductions compared to the other treatments (Table 4).

S. aurantiogriseus VSMGT1014 as a paramorphogen on *R. solani*

Observations of mycelia treated with either 5 μg ml^{-1} of *S. aurantiogriseus* VSMGT1014 crude extract and 5 μg ml^{-1} carbendazim revealed distorted hyphae with abnormal swelling and showed a reduced number of nuclei compared to the control (Fig. 3; Table 5).

Discussion

A number of *Streptomyces* species have been described as bio-control agents against numerous plant pathogens and have shown the potential to produce bioactive compounds as well as to reduce or inhibit mycelia growth of several fungi (Bressan and Figueiredo 2008; Ezziyyzni et al. 2007; Mukherjee and Sen 2006). In recent years, the microbial use of secondary metabolites is gaining importance in crop protection and these metabolites may be a supplement or an alternative to chemical pesticides (Fravel 1988; Shanmugaiah et al. 2010).

In the present study, about 40 % of the 132 actinomycetes isolates tested were found to have antagonistic activity against five fungal plant pathogens; *R. solani*, *M. phaseolina*, *F. oxysporum*, *F. udum* and *A. alternata*. Isolates, VSMGT1014 was found to produce PGP metabolites and to have the largest inhibition zones against the growth of selected fungal pathogens and was therefore used in further studies. There are various mechanisms involved in the disease suppression, one of the primary mechanism of pathogen inhibition as used by plant growth promoting rhizobacteria (PGPR) includes the production of antibiotics, lytic enzymes, volatile compounds and siderophore (Mao et al. 2006). In the present study, strain VSMGT1014 was found to produce IAA, siderophore, volatile substances and lytic enzymes such as chitinase, cellulase, protease, gelatinase, amylase, pectinase and solubilise phosphorus.

In vitro evaluation showed that *S. aurantiogriseus* VSMGT1014 produced several lytic enzymes and secondary metabolites, which might be responsible for strong

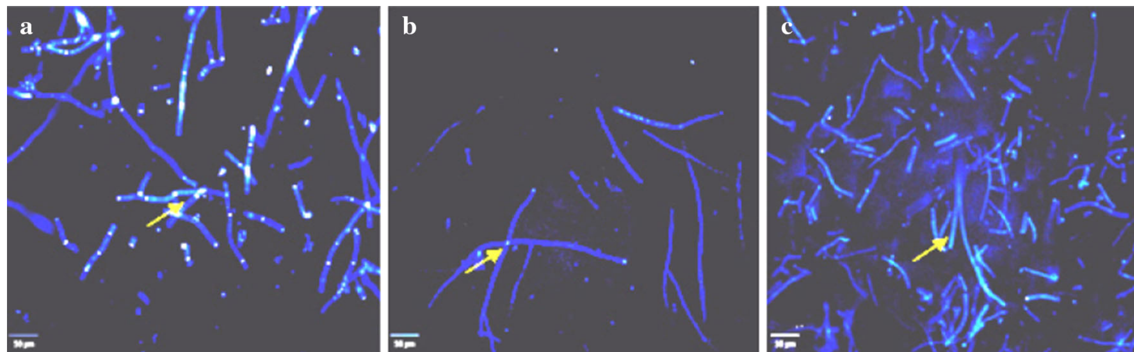

Fig. 3 Hoechst 33258 stained *R. solani* hyphae visualized under high content screening system **a** Untreated fungus (control), **b** treated with carbendazim (positive control) and **c** treated with crude metabolite of *S. aurantiogriseus* VSMGT1014. Arrow indicates in (**a**) nucleation; in (**b**) and **c** de-nucleation and distortion of *R. solani* mycelium

Table 5 Effect of crude metabolites on number of nuclei in *R. solani*

Treatments	Number of nuclei observed	Number of analysed fields
R. solani alone	1,761 ± 4	16
R. solani + crude (5 μg ml^{-1})	265 ± 9	16
R. solani + carbendazim (5 μg ml^{-1})	53 ± 4	16

Values are mean of three replications; ± , standard deviation

antagonistic activity against fungal pathogens observed in our study. Mukherjee and Sen (2006) described lytic enzymes such as chitinase and β-1-3 glucanase produced by *Streptomyces* sp that are used in controlling plant pathogens. The strain VSMGT1014 was found to produce plant growth promoting hormone indole acetic acid. IAA is thought to act a common regulating agent for sporulation and secondary metabolite production by actinomycetes (Matsukawa et al. 2007). Inhibition of *R. solani* mycelial growth by VSMGT1014 culture can be attributed to production of extracellular compounds, such as cell wall degrading enzymes and antibiotics, which damaged and disintegrate the hyphae of *R. solani* observed under the light microscope. The contact of VSMGT1014 with pathogen resulted in the abnormalities such as distortion and hyphal swelling compared to control (Fig. 2).

Microscopic observations revealed disintegration along with some swelling in the pathogen's mycelium which may be due to either lytic enzymes like protease or other secondary metabolites produced by VSMGT1014. The crude extracts from cells-free filtrate of VSMGT1014 has shown antifungal activity towards *R. solani* which was confirmed through a well diffusion assay. The crude metabolites of VSMGT1014 have strong antifungal activity than culture filtrate towards *R. solani* and other fungal pathogens. Interestingly in the detached leaf assay, cells-free culture filtrates showed more activity in terms of control of ShB of rice than the crude metabolites, culture of VSMGT1014 and carbendazim. The percentage of the lesion height for three subsequent intervals (2, 3, 5 days interval) was 22.0, 74.66 and 78.0 % respectively. Similar results were obtained in rice seedlings treated with culture filtrates of *S. globisporous* Jk-1, which control rice blast caused by *Magnaporthe oryzae* was attributed to producing antifungal substances (Li et al. 2011).

Crude metabolites were also observed to affect the formation of nuclei in *R. solani*. Our observation clearly indicated that in the assessment of 16 fields 265, 53 nuclei were observed in samples treated with crude metabolites and carbendazim compared to 1,761 in the control. High content screening showed that 85 and 97 % reduction of nuclei in samples treated with crude metabolites and carbendazim respectively; indicating that isolate VSMGT1014 produced some antifungal metabolites which had very significant effects on the fungal nuclei deformation.

To the best of our knowledge, this is the first report for the control of ShB of rice through detached leaf assay by the strain *S. aurantiogriseus* VSMGT1014 through secretion of both hydrolytic enzymes and secondary metabolites. In addition, this is the first report of crude metabolites of *S. aurantiogriseus* VSMGT1014 controlling of *R. solani* by affecting the cell contents and cell membrane integrity. From the present investigation, it is evident that there are potential bio-molecules in the crude extract of *S. aurantiogriseus* VSMGT1014 which have antifungal properties against a wide spectrum of fungal pathogens. However, further investigations are needed to purify and identify these metabolites from *S. aurantiogriseus* VSMGT1014.

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