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Research Article

ANTIFUNGAL ACTIVITY OF ACTINOBACTERIA WITH A POTENTIAL TO INHIBIT RICE BLAST FUNGUS *MAGNAPORTHE ORYZAE* (ANAMORPH *PYRICULARIA ORYZAE*)

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ABSTRACT

Objective: The aims of the present study were to screen the actinobacteria with high potential ability to produce secondary metabolites that have inhibitory activity against plant pathogenic fungi, *Magnaporthe oryzae*. Production of secondary metabolites was analysis by thin-layer chromatography and bioautography assay.

Methods: Screening and selection of potential *Streptomyces* sp. morphological, cultural, physiological, and biochemical characterization of the screened isolate was carried out. Antifungal compound was confirmed by bioautography assay.

Results: Bioautography method use in this study was found to be antifungal fraction from the crude extract. Antifungal secondary metabolites can be readily located on the plates by observing clear zones where active compounds inhibit fungal growth.

Conclusion: The bioautography assay shows that this isolates can produce antifungal compound. Therefore, this isolate proves to be a promising microbe which can be further studied for its applications a biocontrol agent against rice blast fungi.

Keywords: Secondary metabolites, *Streptomyces violaceoruber*, Rice blast disease, *Magnaporthe oryzae*, Bioautography.

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INTRODUCTION

Rice is a staple food source for more than four billion people worldwide. However, rice is vulnerable to diseases, the most destructive among them being rice blast disease, which is caused by the fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). The plant is prove to the disease at all stages of development, causing annual losses of approximately 30–45% in various rice producing regions. Synthetic fungicides are able to effectively control plant diseases, but some fungicides result in serious environmental and health issues primarily to farmers and consumers. Several studies have been performed for testing of the various compounds produced by *Streptomyces* on *M. oryzae.* The antibiotic Oligomycin A was first isolated from *Streptomyces diastatochromogenes* and was controlling several other plant pathogenic fungi such as *Botrytis cinerea, Cladosporium cucumerinum, Colletotrichum lagenarium, Phytophthora capsici*, *Alternaria alternata*, and *Aspergillus niger* in addition to *M. oryzae* [1-3]. Other compounds such as Pyrroles (Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-) are commonly found in various actinobacterial species [4,5]. Furthermore, Pyrrolo[1,2-a] pyrazine-1,4-dione,hexahydro-3-(phenylmethyl) was found to be able to protect plants from disease causing plant pathogenic fungi and bacteria [6].

Due to the side effects of chemical pesticides, sustainable crop production through eco-friendly, clean, and green management is an essential requirement in the present alarming situation [7]. Therefore, there is growing interest in discovering and developing new, improved fungicides and pesticides based on natural products as well as introducing alternative measures such as biocontrol agents to manage plant diseases and farmers, consumers health.

Actinomycetes are abundant and widely distributed in soil, constituting to about 10–50% of the soil microflora community. Tyc *et al*. [8], Adegboye and Babalola [9], and other researchers have reported them to be important producers of secondary metabolites. The metabolites produced are varied in their biological activities and functions such as antifungal, insecticidal, antibacterial, and anthelmintic activities. Actinomycetes, like other plant growth-promoting microbes, also produce phytohormone [10] and solubilize phosphate [11,12]. *Streptomyces* could indirectly act through increased plant fitness, induction of systemic resistance of plants, and production of bioactive compounds such as antibiotic compounds, siderophore, and some lytic enzymes such as glucanase [13]. Phenazines are a group of nitrogen-containing heterocyclic compounds derived from bacteria of diverse genera such as *Pseudomonas*, *Streptomyces*, *Vibrio,* and Pelagiobacter [14] and their derivatives phenazine-1-carboxamide and phenazine-1-carboxylic acid are considered as important compounds that showed remarkable antifungal activity against disease causing fungal plant pathogen [15].

The bioautography is one of the techniques useful in detecting bioactive compounds from bacterial extracts separated on thin-layer chromatogram. Hence, the objective of this study was to screen secondary metabolites that have inhibitory activity against rice blast plant pathogenic fungi confirmed by bioautography technique.

METHODS

Collection of fungal pathogen and actinobacterial strains

Fungal pathogen collection

M. oryzae-6923 (anamorph *P. oryzae*) was collected from Indian Type Culture Collection.

Actinobacterial strain collection

Actinobacterial cultures were obtained from Actinobacterial Research Laboratory, Department of Microbiology, Periyar University. They included N_{126}, M_{2}, M_{32}, MS_{1-10}, DS_{7-9}, BS_{26}, HS_{3}, BS_{18}, and CSABNC1.

Screening of potential *Streptomyces* strain for bioactive compound production against *P. oryzae*

Primary screening

The 20 actinobacterial cultures were screened for their ability to control the fungal growth. An *in vitro* primary screening method was

used to test the ability of actinobacterial isolates to inhibit the growth of *P. oryzae*. It was done with slight modification of agar plug method of Bharti et al. [16]. Mycelial disks of 6 mm diameter cut out using sterilized cork borer of 5-day-old actively growing pure actinobacterial colony were aseptically inoculated onto the center of potato dextrose agar (PDA) plates [17]. Similarly, *P. oryzae* mycelial disks of 6 mm diameter were cut out from the actively growing pure colony and placed on either side of actinobacteria 3 cm away from central inoculum. All plates were maintained in duplicates and incubated at 28°C for 7 days [18,19].

Inhibition of mycelia growth (%): % Inhibition of mycelia growth = A-B/A × 100, where A is the mycelial growth of pathogen in absence of antagonists; B the mycelial growth of pathogen in presence of antagonists [20]. The isolates exhibiting maximum zone of inhibition against the tested pathogens were selected for secondary screening.

Secondary screening

Actinobacterial isolates selected by primary screening were subjected to secondary screening by agar well diffusion method to test their antagonism upon fungal spore germination and growth. In secondary screening test, the actinobacterial isolates were inoculated in ISP2 medium, pH 7.0, temperature 28°C, and incubated for 7 days. After the incubation period, antifungal activity of crude sample was tested. The spore density was set by spectrophotometer adjusted to 80–85% transmittance at 530 nm with appropriate dilutions to get fungal spore counts of 10^6 spores per ml [21]. The potential isolate screened was mass cultivated in ISP2 medium. This pure culture was further characterized for its identification.

Morphological, cultural, physiological, and biochemical characteristics of strain BS-26

The morphological, cultural, physiological, and biochemical characterization of the isolate was carried out as described in the international Streptomyces project. A sample of the potential screened actinobacteria was prepared according to the protocol described by Srinivasan *et al*. 2014 [22] and external morphology of the isolate studied using scanning electron microscopy (SEM). The micromorphology of the spore bearing hyphae with entire spore chain along with the substrate and aerial mycelium was examined under SEM. The cultural traits of the strain were recorded on different media which included tryptone-yeast extract agar (ISP-1), YMD agar (ISP-2), oatmeal agar (ISP-3), starch inorganic salts agar (ISP-4), glycerol asparagine agar (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7), starch-casein agar, Czapek-Dox agar, and nutrient agar media [23].

The utilization of carbohydrates by the strain was carried out in minimal medium containing different carbon sources at 1% concentration according to the method described by Isik *et al*. [24].

Physiological characterization such as the effect of pH, temperature, and salinity was analyzed. NaCl tolerance of the strain was determined through the technique suggested by Ellaiah *et al*. [25]. Biochemical tests, namely, catalase and coagulase were also evaluated.

Molecular characterization by 16S rRNA sequencing of the potential streptomyces strain producing antifungal compounds

The genomic DNA used for the polymerase chain reaction analysis was prepared from the single colonies grown on the yeast extract malt extract dextrose agar (ISP-2) medium for 7 days. Genomic DNA extracted from the potent strain (BS-26) was isolated employing the DNA purification Kit (Pure Fast Bacterial Genomic DNA purification kit, Helini Biomolecules, India) consistent with the manufacturer protocol. The 16S rRNA gene fragment was amplified using universal primers (forward primer—5- AGAGTTTGATCCTGGCTCAG -3 and reverse primer—5- AAGGAGGTGATCCAGCCGCA -5). The whole Genomic DNA extraction was done [26] and the 16S rRNA sequence of the isolate was subjected to BLAST similarity search tool. The phylogeny (using NCBI) analysis of query sequence with the closely related BLAST result was performed followed by multiple sequence alignment using the program subject sequences of MUSCLE 3.7 [27]. The phylogenetic analysis was performed using the MEGA software package (ver. 6.0) to construct phylogenetic tree based on the neighbor-joining method. The 16S rRNA gene (rRNA) sequence of the strain BS-26 was registered in the GenBank submission.

Extraction of crude secondary metabolites

The clear culture filtrate (1 L) was extracted twice with ethyl acetate (EA) of 1:1 (v/v) and shaken vigorously for 1 h for complete extraction. The EA phase that contains bioactive compound was separated from the aqueous phase. The extracts were pooled and concentrated by rotary evaporation at 40°C. The solvent was evaporated leaving behind the organic residue. The obtained organic residue was subjected to thin-layer chromatography (TLC) separation.

TLC separation

The presence of compounds was analyzed by TLC using TLC aluminum sheets with chloroform and methanol at 9.5: 0.5 ratio (v/v) as solvent system.

The spots were viewed under ultraviolet light for visualization of the spot whose Rf value was calculated using the following formula:

$$Rf = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

Bioautography

Bioautography methods use in this study was employed as a suitable method for direct detecting of antifungal fraction from the crude extract. Antifungal metabolites can be already located on the plates by observing clear zones where active compounds inhibit fungal growth.

Inhibition of fungal growth on chromatographic plate was checked by TLC bioautography system. The chromatogram was kept for evaporation of the solvent. Developed chromatogram was placed on sterile PDA plate. PDA with antagonistic fungal spore suspension was poured on chromatogram. Zone of inhibition was checked after 7 days of incubation.

Antifungal secondary metabolites were located on the plates by observing clear zones where active compound inhibit the fungal growth. Zone of inhibition was checked after incubation [28] period.

RESULTS

Screening of potential actinobacterial strain

Primary screening

Among the 20 actinobacterial isolates that were subjected to antagonistic test by primary screening method, six isolates showed potential inhibition against *M. oryzae* fungal pathogen.

Secondary screening

The six primarily screened actinobacterial isolates were subjected to secondary screening using agar well diffusion method. There was no inhibition zone detected in negative control plate around wells loaded with diluted sterile broth. The inhibition zone in positive control plate with crude bioactive compound of actinobacterial culture was observed to be 22 mm. The zone of inhibition got by the isolate BS-26 was significantly high followed by the isolate BS-18 when compared to all others. From the results of secondary screening, it was observed that BS-26 isolate produced the largest inhibiting zone of diameter 22 mm and BS-18 isolate produced the next largest inhibiting zone of diameter 18 mm (Fig. 1) at 144 h of incubation (from 6^{th} day of inoculation) which stabilized thereon. All the other tested isolates produced zones ranging from 5 to 10 mm diameter during the assays.

Morphological, cultural, physiological, and biochemical characteristics of strain BS-26

Morphological characterization (Fig. 2)

Morphological characters	Response
Colony	Mycelial
Spore chain morphology	Retinaculiaperti
Spore surface	Smooth
Color of aerial mycelium (ISP-2)	Greyish white
Color of substrate mycelium (ISP-2)	Pale yellow

Cultural characteristics

Cultural characteristics of the strain on various ISP and non-ISP media

BS-26 growth was good in ISP-1, ISP-2, ISP-3, ISP-4, and starch-casein agar; moderate in ISP-5, ISP-6, ISP-7, and nutrient agar; and poor in Czapek-Dox agar (Table 1).

Utilization of the carbon sources

Carbon source such as glucose, sucrose, fructose, arabinose, mannose, xylose, rhamnose, and inositol is utilized except raffinose.

Physiological characters

Grams reaction	+
Acid-fast reaction	–
Production of melanin pigment	–
Range of temperature for growth	25–40°C
Optimum temperature for growth	30C
Range of pH for growth	6–9
Optimum pH for growth	7.5
NaCl tolerance	9%

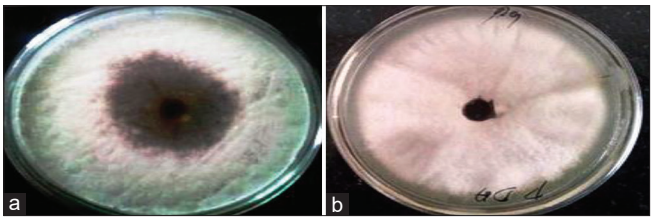

Fig. 1: The inhibition zone of BS-26 compared to the control plate (a) *P. oryzae* controlled by BS-26 (b) control plate

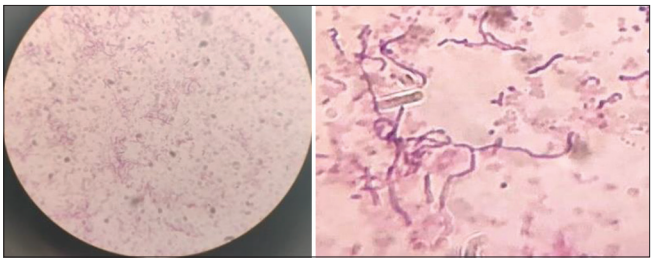

Fig. 2: Micromorphological observation by light microscope

Biochemical characters

Catalase	+
Starch hydrolysis	–
Oxidase	–
Casein hydrolysis	–
Bile esculin	+

The morphological characteristics, cultural characteristics, physiological characteristics, and pattern of utilization of carbon sources of the strain BS-26 revealed that the strain belong to the genus *Streptomyces* [29].

Molecular characterization

A phylogenetic tree based on neighbor-joining method for strain BS-26 along with its closest relatives of the genus *Streptomyces* was given (Fig. 3). The 16S rRNA gene sequence of strain BS-26 has 100% similarity with *Streptomyces violaceoruber*. The sequence had been submitted to the GenBank under the accession number *Streptomyces* MH559817.

Extraction of crude secondary metabolites

The secondary metabolites were extracted from the cell-free culture supernatant using EA. EA extract showed the highest inhibition than other solvent extracts. The obtained organic residue was subjected to TLC separation.

TLC separation

The presence of compounds was analyzed by TLC using TLC silica aluminum sheet with chloroform and methanol at 9.5: 0.5 ratio (v/v) as solvent system (Fig. 4).

Determination of Rf value of the compound.

$$Rf = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

$$\text{The result of Rf value}: \frac{3.5}{5.1} = 0.68$$

Bioautography

Clear zones of inhibition against *M. Oryzae* showed that the culture of *S. violaceoruber* produced secondary metabolites (Fig. 5).

DISCUSSION

The rice blast infection is a major biotic stress caused for the rice crops which is considered as one of the most damaging diseases worldwide and distributed in about 85 countries throughout all continents where the rice crop is cultivated. In India alone, the total loss due to rice blast was 265,000 ton total rice production annually. However, blast under severe epiphytic conditions may result between 70 and 90% losses in isolated fields [30]. Other susceptible factors, that is, high mean temperature values, degree of relative humidity higher than 85–89%, presence of dew, drought stress, and excessive nitrogen fertilization favor epidemic development [31]. Ashkani *et al.* [32] reported that it can cause yield loss between 30 and 50% in large rice producing areas even under favorable environmental conditions. Although fungicides can be used to control rice blast, they generate additional costs in rice

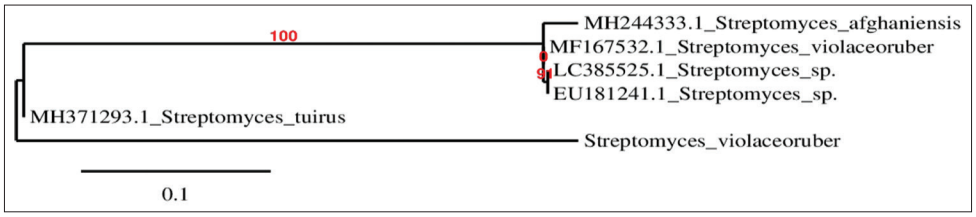

Fig. 3: Highly similar sequence to BS-26

Table 1: Cultural characteristics of the strain on various ISP and non-ISP media

S. No	Medium	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
1	Tryptone-yeast extract agar (ISP-1)	Good	White	Pale yellow	Nil
2	Yeast extract malt extract dextrose agar (ISP-2)	Good	Greyish-white	Yellow	Nil
3	Oatmeal agar (ISP-3)	Good	White	Pale yellow	Nil
4	Inorganic salts starch agar (ISP-4)	Good	White	Pale yellow	Nil
5	Glycerol asparagine agar (ISP-5)	Moderate	White	Pale yellow	Nil
6	Peptone yeast extract iron agar (ISP-6)	Moderate	White	White	Nil
7	Tyrosine agar (ISP-7)	Moderate	White	Yellow	Nil
8	Starch-casein agar	Good	White	Pale yellow	Nil
9	Nutrient agar	Moderate	Greyish-white	Pale yellow	Nil
10	Czapek-Dox agar	Poor	–	–	–

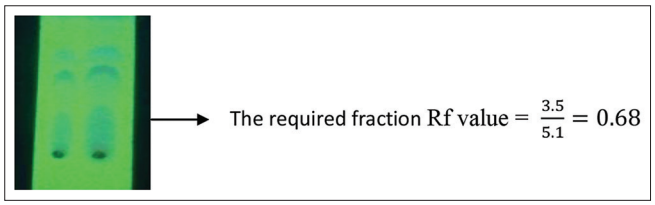

Fig. 4: Thin-layer chromatography separation

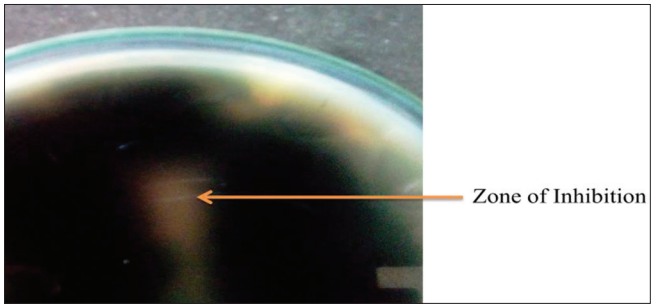

Fig. 5: Bioautography

production and chemical contamination of environment and foods. Therefore, the use of biocontrol solution is needed for rice blast disease management to make significant progress in rice cultivation. Reducing the use of chemical fungicides, pesticides with biocontrol replacement are better solutions to maintain the crop productivity without damaging the ecosystem. Many studies have been conducted on actinobacteria, highlighting the ability of these micro-organisms to promote plant growth and their synergistic effects on plant growth and protection [33]. In the present study, the antifungal activity of secondary metabolites from *S. violaceoruber* was evaluated against *M. oryzae*. The extent of zone formation reflected the inhibition ability of actinobacteria upon the spore germination and mycelia formation of fungal pathogen *M. oryzae.* Bioautography technique was very useful for detection of antifungal compound from the extract on thin-layer chromatograms.

In a previous study [34], it was reported that *S. violaceoruber* had invariably acted as an antagonist to both native and inoculated plant pathogen and helped in plant growth. The antifungal metabolites of present study *S. violaceoruber* can be exploited to indirectly plant growth by inhibiting disease causing pathogens.

CONCLUSION

S. violaceoruber has the ability to produce antifungal compound. Therefore, this isolate proves to be a promising microbe which can be further studied for its applications a biocontrol agent against rice blast fungi. In this study, the antifungal activity of secondary metabolites of *S. violaceoruber* was evaluated against *M. oryzae* and the results suggest that their natural compound could be a potential alternative for controlling rice blast disease.

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AUTHORS' CONTRIBUTIONS

Experimentations and data analysis – R. Thilagam, result interpretation – Dr. N. Hemalatha, actinobacterial strains supply – Dr. R. Balagurunathan and M. Sangeetha.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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