Isolation and characterization of *Streptomyces maritimus* exhibiting *in-vitro* and *in-vivo* antagonistic activity against rice blast pathogen *Pyricularia oryzae*

Bahaderjeet Singh*, Amritpal Mehta, Vasu Mehta and Mahesh V Mahajan

ABSTRACT

A total of 178 microbial cultures were isolated from rhizospheric soil, of which 45 cultures were identified as actinomycetes based on morphology. After primary and secondary screening, the actinomycete GK-121 was found to be a potent biocontrol agent for the rice blast pathogen *Pyricularia oryzae*. The culture was identified using conventional microscopy, Scanning Electron Microscope (SEM) and molecular characterization of the 16S rRNA sequence, and showed 99% similarity with *Streptomyces maritimus*, which was further confirmed by phylogenetic analysis. The culture exhibited a 45 mm zone of inhibition during *in vitro* screening against *P. oryzae*. The greenhouse experiment further proved the effectiveness of the isolate, as the seeds treated with *S. maritimus* showed very low disease incidence in plants inoculated with the pathogen *P. oryzae*, whereas untreated seeds showed high disease incidence. This is the first report to suggest that *S. maritimus* is effective in controlling the rice blast pathogen, *P. oryzae*, *in vitro* and *in vivo.*

Keywords: Rhizosphere, Actinomycetes, Antiphytopathogenic activity, Molecular characterization, Biocontrol

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INTRODUCTION

Rice (*Oryza sativa* L.) is the primary staple food for nearly half of the world's population. Total worldwide production of rice was 500.8 million tonnes 2019/2020 (FAOSTAT, 2020). The rice crop is infected by several kinds of pathogenic microorganisms i.e. bacteria, fungi, viruses etc. The fungal pathogens are major causal agents for large number of diseases in rice. *Pyricularia oryzae*, is a detrimental pathogen, induce heavy losses in rice yield (Kunyosying *et al.*, 2018). At present, this fungus is reported to infect rice crop in at least 85 countries (Howard *et al.*, 2025).

Although chemicals are effective in controlling fungal infections in rice, there are increasing concerns about their improper use, which has resulted in environmental pollution, residual toxicity, and the development of resistance in pathogens (Raman *et al.* 2017). Natural products and microorganisms which are safe to environment and have low toxicity to non-target living organisms, are gaining interest and importance as biocontrol agents and effective substitutes for synthetic fungicides (Komarek *et al.* 2010). Suprapta *et al.* 2012, reported that using microbial antagonists as bio-control agents was effective approach in disease control. According to Pal and Gardener 2006, bioagents control plant pathogens and achieve biological control through competition, antibiosis, and hyperparasitism. *Streptomyces* bacteria are among the microbial antagonists used for managing plant diseases. Ohike, 2018 studied isolation of Actinomycetes that reported strong antifungal activity, showing great potential to

suppress diseases caused by *Rhizochtonia solani* and other phyto-pathogens. In vivo and in vitro studies have reported the potential of *Streptomyces* spp. to control rice blast fungi by multitude of mechanisms such as production of antifungal compounds, cell wall degrading enzymes, siderophores, volatile substances, hyperparasitism etc. (Palaniyandi *et al.* 2013; Faheem *et al.* 2015).

Innovative idea of your study

Soil is the key habitat for actinobacteria, with *Streptomycetes* being a significant part of these populations. Terrestrial Actinobacteria are known for their diverse and intriguing antimicrobial properties (Hasani *et al.*, 2017). The present study focuses on exploration of rhizospheric soil for isolation of actinomycetes strains for the bio control of rice blast. The research seeks to compile the existing knowledge on the bio-control potential of *Streptomyces* consortia against the rice blast fungus, *P. oryzae*.

MATERIAL AND METHODS

The isolation of microorganisms was done in Department of Plant Pathology, Guru Kashi University, during Kharif 2021-2022. *In vitro* experiments were carried out in the particular laboratory in year 2021 while in-vivo experiments were conducted in green house conditions in the year 2022 and 2023.

Isolation of pathogen

The symptoms of Rice Blast were observed and the causal organism was isolated from blast infected plants grown at research field of Guru Kashi University. The infected parts were washed in running tap water. The infected leaves exhibiting typical symptoms were surface sterilized in 4% sodium hypochlorite solution. The small cutting of leaves were transferred to Petri plates containing Oat meat agar (OMA), under aseptic conditions in a laminar airflow and incubated in BOD incubator at 24±2°C. The pathogen was isolated by standard isolation procedure used in the experiment explained by Tuite, in 1969 (Tuite, 1969). The culture was maintained and sub-cultured on PDA slants stored in refrigerator at 4°C for further use.

Isolation of actinobacterial consortia from soil

Samples of soil were collected randomly from root zone of cultivated paddy plants from different regions of Punjab *viz.* Barnala, Mansa, Bathinda, Ludhiana, Raikot, Sardhulgarh, Mullanpur, Malout and GKU farm. Malwa region of Punjab has calciorthids and its pH is 7.8 to 8.5 which is best for *Streptomyces* spp. (Al-Dhabi *et al.*, 2020). The soil samples were air-dried after giving pre-treatments with dry heat (40°C for 1 h) and then preserved in polyethylene bags, after sieving it through 0.8 mm mesh. The serial dilution method was used for isolation of actinomycetes. The 100µL sample was poured on petri plates and incubated at 30°C for 20 days and from the day 7, using various media i.e. Yeast Malt Extract Agar (YMEA), Oat Meat Agar and Nutrient Agar. Isolated colonies were further streaked and incubated at 28°C for one week for the preparation of pure culture at and stored at 4°C in refrigerated.

Antagonistic activity against *Pyricularia oryzae*

Primary screening was carried out using the dual culture method to evaluate the antifungal activity of the isolates. Disks of fungal mycelia, 6 mm in diameter, were positioned 10 mm from the edge of seven-day-old bacterial growth on Yeast Malt Extract Agar (YMA) plates, which were incubated at 28°C. After 3-4 days, the extent of fungal growth inhibition was assessed, and isolates demonstrating antifungal activity were chosen for further testing. Isolates that showed activity in the primary screening were further tested in secondary screening (Singh et al., 2012). The antifungal activity was evaluated by measuring the clear zones of inhibition around wells, recorded in millimeters (mm).

Morphological and molecular characterization of potent culture

On the basis of cultural, morphological, physiological give incomplete information about the classification and taxonomic status of actinobacteria and its comparative analysis by Phylogenetic trees with related strains is used for

actinobacterial systematic. Cultural characteristic determined according to methods recommended by International Streptomyces Project (Shirling *et al.*, 1968). Morphological characteristics of isolates were studied using bright field microscope at 100X using slide culturing technique. Scanning electron microscope (SEM), The aerial mycelium and spore chains grown along the cover slips were fixed with 5% glutaraldehyde for 1 h at room temperature. After fixation cover slips was washed 3 times with distilled water for 5 min each. Post-fixation was done for 2 h in 5% glutaraldehyde followed by rinsing with distilled water for 5 min and this step was repeated thrice. Dehydration was carried out using different concentrations of ethanol (50%, 70 %, 80%, 95 and 100%) for 10 min each. The sample was placed in a vacuum desiccator and gold-coated with an E-1010 Ion Sputter Coater. It was subsequently observed under secondary electron imaging mode using a Hitachi S-3400N Scanning Electron Microscope. After desiccation, cells were coated with gold and observed under SEM.

Molecular characterization

The culture was grown in glucose broth for 96 h at 28°C at 120rpm and harvesting of biomass was done at 10000rpm for 15min using centrifuge. The cells were washed with 0.9% NaCl and mixed with 550µl of extraction buffer (TE) (50 $mmoll^{-1}$ Tris–HCl, pH 8.0; 700 $mmoll^{-1}$ NaCl; 10 $mmoll^{-1}$ EDTA, 1 % (v/v) β- mercaptoethanol and 10% (w/v) SDS). The DNA in the aqueous phase was deproteinated and purified by repeatedly extracting with equal volumes of a saturated phenol, chloroform, and isoamyl alcohol (PCI) mixture (25:24:1). The resulting DNA pellet was rinsed with 70% (v/v) ethanol, air-dried, dissolved in 50 µl of sterilized double-distilled water, and stored at 4°C (Singh *et al.* 2012).

PCR amplification

The DNA amplification was done using 27f (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492r (5'-AGAAAGGAGGTGATCCA GGC-3') primers. PCR amplification was conducted in 0.2 ml PCR tubes with a Mastercycler Personal (Eppendorf). Each 50 µl PCR reaction mixture consisted of 25 µL of PCR master mix (Genei, Bangalore, India), 2.5 µl of DMSO, 1 pmol of each primer, and 100 ng of template DNA. The amplified products were resolved by electrophoresis on a 1.4% (w/v) agarose gel containing ethidium bromide, using 1× TAE buffer at 70 V. The PCR products were then sequenced at the Central University of Bathinda, Punjab.

Phylogenetic analysis

Phylogenetic analysis was performed with NCBI to determine phylogenetic neighbors and compute pairwise 16S rRNA gene sequence similarities. Phylogenetic trees were constructed using neighbor-joining and maximum-parsimony methods, with bootstrap values calculated from 1000 replications, employing MEGA 5 software (Tamura *et al.*, 2011).

***In vivo* antagonistic activity testing**

The *in vivo* antifungal activity of the *Streptomyces maritimus* strain was evaluated under greenhouse conditions to check its efficacy. The experiment was performed in pots undertaking following treatments of rice seeds cv. PR114 (Susceptible to rice blast), the seeds were sown in the pots containing sterilized soil and humus of decayed leaves (3:1 w/w) in greenhouse conditions.

The carrier materials was studied that talc and suspension and its composition and mixing process of materials as follows: (a) 300 ml suspension of bacterial isolates (10^9 CFU/ml) (300ml suspension of YME broth was centrifuged at 10000rpm and the cells were dissolved in 300ml sterilized distilled water), 1 kg of talc, 10 g of carboxymethyl cellulose (CMC), 15 g of CaCO3. Before transplanting, the rice seedlings were immersed in talc overnight and then treated with a suspension for 3 hours. *P. oryzae* inoculation was achieved by spraying spores at a density of 5.0×10^8 spores/ml, with the plants maintained under high humidity conditions. Rice plants that were not treated with *P. oryzae* but inoculated with *Streptomyces maritimus* served as the positive control, while those inoculated with *P. oryzae* but without bacterial suspension were used as the negative control. Data were collected from eight plants (with 4-5 leaves per plant) for each

treatment (Suryadi *et al.,* 2013). Observations were made at two-week intervals, following the disease assessment guidelines outlined in the standard evaluation system of IRRI (1996). Then the severity of blast is calculated using the formula:

$$\mathbf{DS} = \sum \frac{n \times v}{N \times V} \times 100$$

DS = disease severity
n = number of leaves infected by blast
v = value score of each category attack
N = number of leaves observed
V = value the highest score

Statistical analysis

The experimental data were statistically analyzed at the using SPSS. Observations of percent disease inhibition and intensity were converted into values of arcsine and square transformation for statistical analysis.

RESULTS AND DISCUSSION

Isolation and identification of test fungi

The fungal strain was isolated from infected leaves, the fungus was found to be slow growing. After 24 days of incubation of fungus in BOD at 24+2°C, more conidia were produced as compared to the cultures at 6, 12 and 18 days of incubation, conidial production increased with increasing in incubation period. The grayish black smooth colonies were observed on OMA media, long pyriform conidia and dense mycelium.

Isolation of actinomycetes from soil and analysis of antifungal activity

Total 200 bacterial and actinomycete cultures have been isolated, 178 cultures were isolated from rhizospheric/soil while 22 cultures were isolated from non-rhizospheric soil. On the basis of morphological observations 45 actinomycete cultures were selected for primary screening. Twenty two isolates showed medium to high antagonistic activity while isolates GK-52, GK-65 and GK-121 found to be potent culture and selected for secondary screening (Table 1). During secondary screening highest zone of inhibition of 45mm was observed in GK-121 while zone of inhibition of 33mm and 28mm was observed in GK-65 and GK-52, respectively (Fig. 1 a, b&c).

Table 1. Zone of inhibition, exhibited by active isolates during secondary screening.

Active Isolates	Zone of inhibition (mm)
GK 33	10 add SD or SE
GK 47	06
GK52	33
GK56	15
GK65	28
GK 75	12
GK77	13
GK106	10
GK114	13
GK 116	15
GK121	**45**
GK123	11
GK124	22
GK125	05
GK145	16
GK148	10
GK150	15
GK166	15
GK169	13
GK170	18
GK182	28
GK192	23

S.E. ± 2.06; C.D. at 5% 2.40

Identification of actinomycete culture GK-121

Scanning electron microscopy

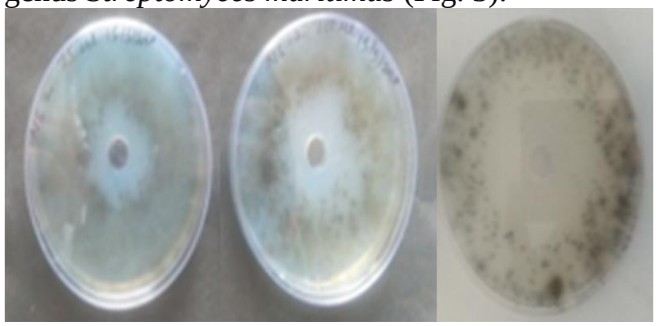
The SEM revealed the detailed morphology of GK-121, the intact spore structure and ornamentation with minimum distortion and disturbance was visible at higher magnifications. Scanning electron micrographs of actinomycetes depict a thick, single layer of cells at the outer edge of the colony, arranged in adjacent uniform chains. Observations of the developing colonies showed that new cells formed at the colony's center divided, leading to the elongation of the chains and a curved configuration (Fig. 2 a, b & c).

Figure.1. Morphology of *Pyricularia oryza*e on petriplate and under microscope (a) Growth on OMA medium (b & c) Morphology of spores and mycelium.

a b c

Molecular characterization

The 16S rRNA sequence obtained was compared to the nucleotide sequence database at the National Center for Biotechnology Information (NCBI) using the BlastN program. The analysis revealed that the actinomycetal isolate GK-121 closely matches the genus Streptomyces. Genetic relationships of strain GK-121 were assessed by amplifying the 16S rRNA gene via PCR, resulting in an amplified sequence of 1213 base pairs. The newly identified sequence was subsequently submitted to GenBank with accession number MW172253. The sequence of strain GK-121 showed 99.9% similarity to *Streptomyces maritimus*. Phylogenetic analysis and alignment with homologous nucleotide sequences confirmed that strain GK-121 is most closely related to the genus *Streptomyces maritimus* (Fig. 3).



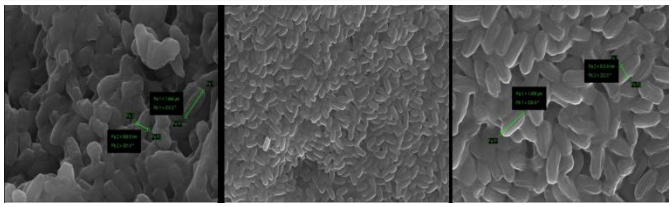
a b c

Figure 2. Well diffusion assay of potent actinomytcetes culture showing zone of inhibition (a) GK-52 (b) GK-65 (c) GK-121.

Effect of Bacterization on occurrence of Disease Incidence

Table 2. The occurrence of percent disease incidence of blast on rice plants in green house experiment. The experiment was conducted in triplicates and mean value was calculated. Under green house condition, the seedlings inoculated with *P. oryzae* fungus showed 60-91% of disease incidence while the rice plants treated with both *S. maritimus* and *P. oryzae* showed 5-10 % disease incidence. The Plant which is untreated (without *P. oryzae* and *S. maritimus)* also recorded with 20-35% of disease incidence. The disease increases with increase in time, in *P. oryzae* inoculated plants disease incidence was 60% after 15 days while it increases to 91% after 45days, similar trend was noticed in other two treatments as well. The incidence of disease increases with time as all the treatments indicates the disease incidence was less after 15 days while maximum after 45 days (Table 2). The results of *S. maritimus* were found promising as it reduces the disease incidence approximately by 70%. The treated seedlings remained healthy up to the last day (45th day) of observation along with the control (Fig. 4).

The evolutionary distances were computed using the Kimura two-parameter method and were in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair. Evolutionary analysis was conducted in MEGA5.



a b c

Figure 3. Scanning Electron Micrograph (SEM) of actinomycete isolate *Streptomyces maritimus* GK-121.

Table 2. The occurrence of percent disease incidence of blast on rice plants in green house experiment. The experiment was conducted in triplicates and mean value was calculated.

Treatments	**Days after disease incidence (%)**		
	15 days	**30 days**	**45 days**
Untreated Control	30	35	45
Pathogen Inoculated with *P. oryzae*	62	75	91
Pathogen + GK-121	3	5	10

In current scenario agriculture system is facing several major plant diseases in spite of using resistant cultivars, chemical pesticides and various agricultural practices. Rice blast disease is one of major cause of huge losses all over the world, mainly chemical based fungicides are in use to control the disease. The continuous and indiscriminate use of agrochemicals, globally affects the consumer health and environment. Therefore, it is very important to find alternative solutions to reduce the use of chemical pesticides. In queue of this, Biological control by using microorganisms has gained the attention of researchers as safe as eco friendly substitute of agrochemicals The current study revealed that the non-rhizospheric soil contain less number 22 of cultures as compared to rhizospheric soils contain more number 178 cultures. These findings suggest that the rhizosphere provides a biological niche for a wide variety of microorganisms, owing to the substantial organic matter input from plant roots and root exudates. to soils that are not associated with the rhizosphere (Crawford *et al*. 1993). During primary screening the actinomycetes were selected and secondary screening was used to identify best culture among the isolated cultures.

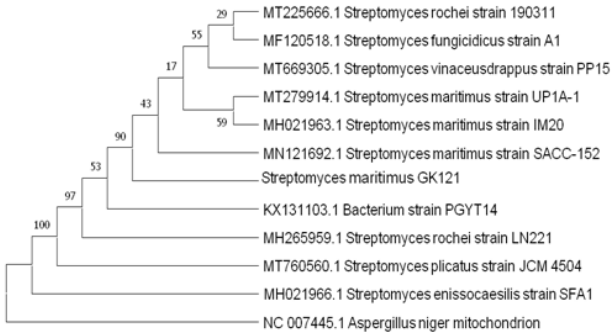


Figure 4. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

As a result, it supports almost twice as many actinobacteria isolates as compared

(a) (b) (c)

Figure 5. Observation of rice plants after 45 days (a) inoculated with *P. oryzae* (b) uninoculated plant (c) treated with *Streptomyces maritimus* GK-121+ *P. oryzae*.

The culture was identified using conventional microscopy, SEM, TEM and then by molecular methods and phylogenetic analysis which revealed that the culture has maximum similarity with *Streptomyces maritimus*. The actinomycetes, especially streptomycetes, are known to produce different biologically active metabolites (antibiotics, enzymes, plant growth regulators etc.) of commercial importance in agricultural industry (references). Ningthoujam, reported actinomycetes

showing great potential to suppress diseases caused by *Rhizochtonia solani* and other phyto-pathogens (Ningthoujam *et al.,* 2009). *Streptomyces rimosus* MY02 and *Streptomyces malachitofuscus* CTF9 have been documented for their production of antifungal compounds (Yu *et al.,* 2017). Furthermore, *Streptomyces coeruleorubidus* was found to produce the maximum amount of bioactive metabolites at 35°C (Bundale *et al.,* 2015). Augustine et al. (2005) observed that *Streptomyces rochei* AK 39 is able to produce various antifungal compounds at optimum agitation and aeration.

The *in-vivo* studies revealed that the seeds treated with *Streptomyces maritimus* shown promising control of rice blast in green house pot experiment. The disease incidence is maximum in *P. oryzae* inoculated plants and minimum in both *Streptomyces maritimus and P. oryzae* treated plants. Various studies have been conducted previously for the use of *Streptomyces* bacteria as biocontrol agents in agriculture. The several strains of Streptomyces are used to control plant diseases caused by *Fusarium* spp., *P. oryzae, Colletotrichum gloeosporioides* and *Microcyclusule* (Villarraga *et al.* 2017). *Streptomyces* sp. Di-944 is another wettable formulation which prevents tomato damping-off caused by *Rhizoctonia solani* (Sabaratnam *et al.* 2002). In recent years, the use of crude secondary metabolites produced by *Streptomyces* spp. are also gaining importance in crop protection and these metabolites may be a supplement or an alternative to chemical pesticides (Harikrishnan *et al.,* 2014).

The results of the present study endorse the potential of *Streptomyces maritimus* can be used as biocontrol agent. Though actinomycetes are extensively reported as bioconrol agents but this is the first report which suggests that *S. maritimus* GK-121 is effective to control Rice blast pathogen *P. oryzae in-vitro* as well as *in-vivo.* The field experiments and plant growth promoting studies of the culture are in progress.

Conflict of interest

The authors declare no competing interests.

Author contributions

Bahaderjeet Singh conceptualize the idea of isolation of actinomycetes from rhizospheric and non-rhizospheric soil and evaluation of their antagonistic activity against *P. oryzae*. Mr. Mahesh did isolation and research work in the field as well as in the lab. Vasu Mehta and Amritpal Mehta collected the literature and edited the article.

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Bahaderjeet Singh*, Amritpal Mehta, Vasu Mehta and Mahesh V Mahajan

Faculty of Agriculture, Guru Kashi University, Talwandi Sabo, Punjab, India

*Corresponding author

E-mail: drbahaderjeetsingh@gku.ac.in

Contact no. 9417552552