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IN VITRO ANTIFUNGAL ACTIVITY OF ACTINOBACTERIA AGAINST PADDY FUNGAL PATHOGENS *ATHELIA ROLFSII* AND *COCHLIOBOLUS LUNATUS*

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ABSTRACT

The present study deals with the population of actinobacteria from mangrove environment of Ennore, Kovalam, Mamallapuam, Palaverkadu, East coast of Tamil Nadu, India. Totally 85 actinobacteria species belonging to different genera were isolated from mangrove environment of marine soils. The potential of actinobacteria against selected paddy fungal pathogens such as *Athelia rolfsii* and *Cochliobolus lunatus* and to find out the efficient antifungal activity of actinobacteria against paddy fungal pathogens. To evaluate the solubility of the antifungal compounds using different solvents such as chloroform, ethyl acetate, methanol and determine the antifungal efficacy of selected potential actinobacteria by agar well diffusion method. The isolated actinobacteria strains were then screened with regard to their potential of antifungal activity.

KEYWORDS: Mangrove environment, Actinobacteria, Paddy fungal pathogens.

INTRODUCTION

Fungal pathogens can be exploited as biological agents for the management of agricultural pests and diseases (Evans, 1999). The diversity of fungal spores which cause diseases to this crop which leads to the decrease in food production (Anonymous, 2005).

The Worldwide efforts in the search of natural products for the crop protection market have progressed significantly and actinomycetes, especially those belonging to the genus *Streptomyces*, appear to be good candidates to find new approaches to control plant diseases (Behal, 2000).

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces* are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Anitha and Rebeeth, 2009).

Fungal diseases in agricultural crops possess a great challenge. The currently used fungicides are either less effective or of great environmental concern. Hence, it is necessary to develop environmentally safer and potent fungicides of natural origin. Microbial metabolites have been expected to minimize the deleterious side effects caused by synthetic fungicides. It neither eliminates the pathogen nor the disease, but brings them into natural balance (Ramanathan *et al.,* 2002). Microorganisms as biological control agents have high potential to control plant pathogens and no effect on the environment or other non-target organisms (Sharifi *et al.*, 2007).

Athelia rolfsii is a corticioid fungus in the family Atheliaceae. It is a facultative plant pathogen and the causal agent of "Seedling blight" disease in rice. Seedling blight caused by the fungus *Athelia rolfsii* Sacc. (teleomorph *Sclerotium rolfsii* (Curzi) C.C. Tu & Kimbrough) is a serious disease affecting diverse crops grown around the world especially in tropical and subtropical regions.

Cochliobolus lunatus is a fungal plant pathogen that can cause black kernal disease in rice. The anamorph of this fungus is known as *Curvularia lunata*, while *Cochliobolus lunatus* denotes the teleomorph or sexual state. *Cochliobolus lunatus* has a widespread distribution, though it is especially prevalent in the tropics and subtropics (Nelson and Haasis, 1964). Infection is caused by air borne conidia and ascospores, however, sclerotioid *C. lunatus* can also survive in the soil (Cunha *et al*., 2013, Zhang *et al.,* 2013) Possible cause of black kernels in rice (*Oryzae sativa*).

MATERIALS AND METHODS

Diversity of actinobacteria

Totally 85 actinobacteria species belonging to different genera were isolated from mangrove environment of marine soils.

Species composition of actinobacteria

In general, among the 30 genera were recorded, the genus of *Streptomyces* (19) was dominant followed by *Nocardiopsis* (7 strains each), *Nocardia* (6 strains each), *Actinomadura* (3 strains

each), *Saccharothrix* (4 strains each), *Saccharopolyspora* (4 strains), *Streptoverticillium*(4 strains each), *Actinobispora* (3 strains each), *Actinokineospora* sp (3 strains each), *Microtetraspora* (3 strains each), *Pseudonocardia* (3 strains each), *Terrabacter* (3 strains each), *Catellospora* (2 strains each), *Jonesia* (2 strains each), *Planomonospora* (2 strains each), *Thermoactinomycetes*(2 strains each), *Actinopolyspora* sp., *Amycolata* sp., *Glycomyces* sp., *Microbispora* sp., *Micropolyspora* sp., *Promicromonospora* sp., *Spirillospora* sp., *Thermomonospora* sp., *Planobispora* sp., *Kineospora* sp., *Micromonospora* sp., *Kitasatosporia* sp., *Streptosporangium* sp., *Actinosynnema* sp. and *Rhodococcus* sp. All other genera were represented by one strain each.

Isolation of *Athelia rolfsii* from infected Paddy grains

The fungal infected paddy grains were collected from Pattukkottai, Thanjavur district Tamil Nadu, India to screen *Athelia rolfsii* and *Cochliobolus lunatus.*

Preparation of potato dextrose agar medium (Warcup, 1950)

The potato dextrose agar medium was prepared and autoclaved at 121°C for 20 minutes at 15 lbs pressure. The medium was incorporated with 50 mg/ml Streptomycin Sulphate solution and mixed well to prevent the bacterial contamination. The surface sterilized infected paddy grains were inoculated in the medium and the plates were incubated at 28±2^{o} C for 5 days.

Observation

The colonies growing on plates with different morphology were counted separately. The fungal cultures were then transferred, subcultured and the pure cultures were maintained on PDA medium. A portion of mycelium of the representative colonies were picked up with the help of a pair of needles and semi permanent slides were prepared using lactophenol cotton blue (20g -Phenol crystals; 20g-lactic acid (SG1 21); 40g-Glycerine; 20ml - water; Cotton blue - a few drops). The slide was observed under a compound microscope. Morphology of the individual fungal species was also recorded using Nikon phase contrast microscope (Nikon, Japan).

Preliminary screening of actinobacteria metabolites against *Athelia rolfsii* and *Cochliobolus lunatus*

One mg of mycelium of actinobacteria was crushed in broth to extract bioactive compounds. Then it was filtered by Whatman No.1 filter paper. After filtration these actinobacteria extract were separately stored in test tubes.

Preliminary screening

The antifungal activities of the selected actinobacteria were tested against the *Athelia rolfsii* and *Cochliobolus lunatus*. The 20 ml of sterilized Potato dextrose agar medium was poured into each sterile petriplates and allowed to solidify. The test fungal pathogen was evenly spread over the PDA media by using a sterile cotton swab. Then a well of 6 mm was made in the medium by using sterile cork borer, 200µl of each actinobacteria crude extract were transferred into separate well. Then these plates were incubated at 28 ±2^{o}C for 48-72 hours. After incubation the results were observed and the diameter of inhibition zone was measured around the each well.

Antifungal activity of potential actinobacteria

Preparation of actinobacteria solvent extract

The potential actinobacteria strains were selected by preliminary screening and inoculated in starch casein broth and incubated at 28 ±2^{o}C for 7 days. The biomass were crushed with solvents (chloroform, ethyl acetate and methanol) using mortar and pestle. The extracts were centrifuged at 5,000 rpm for 10 minutes. The supernatant was taken and used for antifungal assay.

Preparation of fungal inoculum

The young fungal inoculum were prepared and used for the present study. The Potato dextrose broth (PDB) was prepared and poured and into tubes. The isolated pure fungal strains were inoculated into the tubes using the inoculation loops and were incubated at 28 ±2^{o}C for 48-72 hours.

Antifungal assay method

In the freshly prepared and sterilized Potato dextrose agar medium, Streptomycin was added to prevent the bacterial contamination and mixed well. The medium was poured into each petriplate and allowed to solidify. The test fungal cultures were evenly spread over the appropriate media by using sterile cotton swab. Then 6 mm wells were made in the medium by using sterile cork borer and 200µl of the actinobacteria extracts (Chloroform, ethyl acetate, methanol) were poured into separate wells. Then these plates were incubated at 28 ±2^{o}C for 48-72 hours. After the incubation period the results were observed and the diameter of inhibition zone around the each well was measured.

RESULTS AND DISCUSSION

The present research has been initiated to identify the novel actinobacteria from mangrove environment were collected from at month wise. Totally 85 actinobacteria were isolated from four different stations using starch casein agar medium (Kokare *et al*., 2004; Dhanasekaran *et al*., 2008; Basavaraj *et al*., 2010; Kumar *et al*., 2010; Usha *et al*., 2010; Kalyani *et al*., 2012).

The marine soil samples were collected from mangrove environment of four different geographical regions such as Ennore, Kovalam, Mamallapuram, Palaverkadu, East coast of Tamil Nadu, India. The diversity of actinobacteria showed the existence of 85 species belonged to 30 genera were recorded. All the 85 actinobacteria were identified at a generic level based on the colony morphology, aerial mycelium, colour, nature of colony and microscopic characterization.

Elamvazhuthi and Subramanian, (2013) was reported that the 15 isolates of actinomycetes were isolated from upland paddy of Jeypore, Odissa. They were screened for their antagonistic activity against four different fungal plant pathogens namely *Rhizoctonia solani*, *Helminthosporium oryzae*, *Curvularia lunata* and *Fusarium oxysporum* by dual plate assay. The antagonistic effects were more prominent after two days in *R. solani* and three days in the other fungi.

Sclerotium rolfsii Sacc, *Athelia rolfsii* (Curzi) Tu & Kimbrough causes the disease known as southern blight in a wide variety of crops. *Sclerotium rolfsii* forms brownish sclerotia that can survive in soil for long periods, frequently tolerating biological and chemical degradation due to the presence of melanin in the outer membrane (Chet, 1975). Among the methods employed to manage *S. rolfsii* are the following: fungicide applications, solarization and use of antagonistic microorganisms, deep plowing, crop rotation and incorporation of organic and inorganic residues (Punja, 1985; Swaminathan *et al.*, 2007; Compant *et al.*, 2005).

28 fungi detected in 97 samples of discoloured rice seed from 4 agro climatically different areas, 16 were associated with seed from Bihar, 23 from Jammu, 21 from Andhra Pradesh and 23 from Orissa. *Drechslera oryzae* (*Cochliobolusmiya beanus*) followed by *Curvularia lunata* (*Cochliobolus lunatus*) predominated in Bihar; *Phoma* sp. followed by *C. miyabeanus* and *Alternariacalternate in* Jammu; *Phoma* sp., *C.lunatus* and *Nigrospora* (*Khuskia*) *oryzae* in Andhra Pradesh and *C. lunatus* dominated in Orissa. *C. lunatus* was predominant in 81 of the samples (Misra and Dharam, 1988).

In the present investigation, the crude extracts of selected 30 actinobacteria species was used to check the preliminary antifungal activity against paddy pathogenic fungus, *Athelia rolfsi*. The crude extracts of actinobacteria species shows different zone of inhibition against *Athelia rolfsii*. The maximum zone of inhibition was due to *Streptomyces griseus* KSPE17 (13.2±1.6), followed by *Streptoverticillium cinnamoneum* KSPK12 (9.8±2.0), *Spirillospora* sp. KSPE20 (8.3±1.0), *Streptomyces* sp. KSPK17 (8.1±1.3), *Actinokineospora* sp. KSPM5 (7.9±1.8), *Nocardia* sp. KSPM11 (7.6±2.0), *Thermoactinomycetes* sp. KSPK23 (7.2±1.5), *Kineospora* sp. KSPK5 (6.5±1.0), *Nocardiopsis* sp. KSPE10 (6.2±1.4), *Streptomyces anulatus* KSPP16 (5.5±0.8); *Terrabacter* sp. KSPK21 (5.3±0.9) (Table 1).

The crude extracts of actinobacteria species shows different zone of inhibition against *Cochliobolus lunatus*. The maximum zone of inhibition was due to *Streptomyces griseus* KSPE17 (8.2±1.4) followed by *Thermoactinomycetes* sp. KSPK23 (7.7±0.9), *Streptomyces* sp. KSPK17 (7.5±0.8), *Streptomyces anulatus* KSPP16 (7.2±0.9 each); *Spirillospora* sp. KSPE20, *Streptoverticillium cinnamoneum* KSPK12 (7.1±1.2 each); *Kineospora* sp. KSPK6, *Streptomyces microflavus* KSPE15(6.5±1.2), *Terrabacter* sp. KSPK21 (6.4±1.3), *Actinokineospora* sp. KSPM5 (5.6±1.5), *Nocardiopsis* sp. KSPE1(5.4±0.8), *Kitasatosporia* sp. KSPP5(5.3±1.1), *Streptoverticillium* sp. KSPE19 (5.2±1.5) (Table 1)..

The methanol extract of *Streptomyces griseus* KSPE17 showed very promising result in antifungal activity by exhibiting prominent zone of inhibition against *Athelia rolfsii* (16.3±1.5) and *Cochliobolus lunatus* (14.1±2.5) respectively (Table 2 & 3).

The ethyl acetate extract of selected strains exhibited moderate activity and least activity was showed by chloroform extracts. *Sclerotium rolfsii* is a pathogenic fungus, infects about 500 plant species of about 100 families. *Streptomyces species* MTCC4 showed remarkable activity against *Sclerotium rolfsii*. The culture broth of *Streptomyces species* MTCC4 showed maximum antifungal activity methanol extract (Verma *et al*., 2010).

Table 1. Screening of antifungal activity of selected actinobacteria isolates against *Athelia rolfsii* and *Cochliobolus lunatus*

S.No	Name of actinobacteria	Zone of inhibition (mm) *Athelia rolfsii*	Zone of inhibition (mm) *Cochliobolus lunatus*
1.	*Actinokineospora* sp. KSPM5	7.9±1.8	5.6±1.5
2.	*Catellospora* sp. KSPP4	-	-
3.	*Kineospora* sp. KSPK5	6.5±1.0	7.1±2.3

4.	*Kitasatosporia* sp. KSPP5	-	5.3±1.1
5.	*Micromonospora* sp. KSPK6	-	-
6.	*Microtetraspora* sp. KSPM9	-	-
7.	*Microtetraspora* sp. KSPM10	-	-
8.	*Nocardia* sp. KSPM11	7.6±2.0	-
9.	*Nocardiopsis* sp. KSPE10	6.2±1.4	5.4±0.8
10.	*Nocardiopsis* sp. KSPP7	-	-
11.	*Nocardiopsis* sp. KSPP8	-	-
12.	*Pseudonocardia* sp. KSPK3	-	-
13.	*Pseudonocardia* sp. KSPM13	-	-
14.	*Planobispora* sp.KSPP11	-	-
15.	*Planomonospora* sp. KSPP12	-	-
16.	*Rhodococcus* sp. KSPM14	-	-
17.	*Spirillospora* sp. KSPE20	8.3±1.0	7.2±1.9
18.	*Streptomyces griseus* KSPE17	13.2±1.6	8.2±1.4
19.	*Streptomyces microflavus* KSPE15	-	6.5±1.2
20.	*Streptomyces* sp. KSPK16	-	-
21.	*Streptomyces* sp. KSPK17	8.1±1.3	7.5±0.8
22.	*Streptomyces* sp. KSPK18	-	-
23.	*Saccharothrix* sp. KSPM16	-	-
24.	*Streptomyces anulatus* KSPP16	5.5±0.8	7.2±0.9
25.	*Streptoverticillium* sp. KSPE19	-	5.2±1.5
26.	*Streptoverticillium* sp. KSPP21	-	-
27.	*Streptoverticillium cinnamoneum* KSPK12	9.8±2.0	7.1±1.2
28.	*Thermomonospora* sp. KSPE21	-	-
29.	*Terrabacter* sp. KSPK21	5.3±0.9	6.4±1.3
30.	*Thermoactinomycetes* sp. KSPK23	7.2±1.5	7.7±0.9

- Absence of antifungal activity

Table 2. Antifungal activity of selected actinobacteria against *Athelia rolfsii*

S.No	Name of actinobacteria	Zone of inhibition (mm)		
		Chloroform	Ethyl acetate	Methanol
1.	*Actinokineospora* sp. KSPM5	-	-	8.5±1.3
2.	*Kineospora* sp.KSPK5	-	-	9.1±1.5
3.	*Nocardiopsis* sp. KSPE10	-	5.5±0.5	8.2±1.4
4.	*Nocardia* sp. KSPM11	-	-	7.4±1.8
5.	*Spirillospora* sp. KSPE20	8.6±1.0	6.4±1.0	7.6±0.9
6.	*Streptomyces anulatus* KSPP16	-	-	8.2±1.9
7.	*Streptomyces griseus* KSPE17	5.5±1.6	8.6±1.3	16.3±1.5
8.	*Streptomyces* sp. KSPK17	-	-	8.2±2.0
9.	*Streptoverticillium cinnamoneum* KSPK12	-	5.2±1.3	12.4±2.4
10.	*Terrabacter* sp. KSPK21	-	-	6.1±1.9
11.	*Thermoactinomycetes* sp.KSPK23	-	-	7.4±1.5

- Absence of antifungal activity

Table 3. Antifungal activity of selected actinobacteria against *Cochliobolus lunatus*

S.No	Name of actinobacteria	Zone of inhibition (mm)		
		Chloroform	Ethyl acetate	Methanol
1.	*Actinokineospora* sp.KSPM5	-	5.2±1.5	9.7±1.4
2.	*Kineospora* sp. KSPK5	-	6.4±1.4	8.2±1.2
3.	*Kitasatosporia* sp. KSPP5	5.7±1.2	6.2±2.0	6.0±1.4
4.	*Nocardiopsis* sp. KSPE10	-	-	5.1±2.1
5.	*Spirillospora* sp. KSPE20	-	-	5.3±1.1
6.	*Streptomyces griseus* KSPE17	5.3±1.2	5.5±1.5	14.1±2.5
7.	*Streptomyces microflavus* KSPE15	-	-	6.4±1.8
8.	*Streptomyces* sp. KSPK17	-	5.9±1.5	7.6±2.5
9.	*Streptomyces anulatus* KSPP16	-	-	7.1±1.2
10.	*Streptoverticillium* sp. KSPE19	-	6.0±2.0	7.6±1.7
11.	*Streptoverticillium cinnamoneum* KSPK12	-	-	8.7±1.3
12.	*Terrabacter* sp. KSPK21	-	-	8.1±1.6
13.	*Thermoactinomycsetes* sp. KSPK23	-	-	8.5±1.3

- Absence of antifungal activity

Findings of the present study conclude that mangrove environment actinobacteria are the potential ecosystem for antagonistic actinobacteria which deserves for bioprospecting. This study investigated that *Streptomyces griseus* KSPE17 exhibited higher activity against paddy fungal pathogens. This *Streptomyces griseus* KSPE17 have a potential to be included in researches of new preparations with antifungal action also for plant protection.

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