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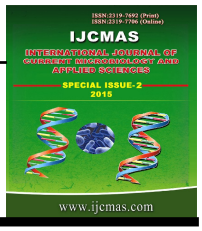

Original Research Article

Isolation and Characterization of *Actinomycetes* from Rhizosphere Soil of Different Plants for Antiphytopathogenic Activity and Stress Tolerance

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A B S T R A C T

Plant diseases need to be controlled to maintain the quality of food, feed, and fiber produced by growers around the world. Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year. In this study we have isolated and characterized actinomycetes from rhizosphere of some medicinal plants and soil near brick factory. These isolates were screened for their antifungal activities against five selective pathogens of plants. Totally 10 isolates of actinomycetes were obtained from all soil samples. These were characterized by slide culture technique, salt tolerance, growth at different pH and temperature, biochemical characterization and enzymatic activity. Agar streak method and dual culture bioassay was used for screening of antifungal activity of these isolates. Fungal pathogens used in this study are *F. oxysporium*, *F. soloni*, *A. alternata*, *A. flavus*, and *C. gloeosporioides.* Out of 10 isolate of Actinomycetes, isolate (A3) has showed wide spectrum activity against all the fungal pathogen. Dual culture assay showed significant activity, 86.66 % inhibition against *C. gloeosporioide* by A3 and 70.49% inhibition against *A. alternata* by BF5 isolate. A3 and BF5 tolerate salt concentration (NaCl) up to 5% and 7.5 % respectively. These two isolates were identified as *Streptomyces* spp. based on morphology, microscopy and 16s rDNA partial sequencing. This preliminary in vitro study revealed actinomycetes as potential antifungal candidates. Stress tolerance tests demonstrate their application in ecologically diverse areas. This work leads and support use of actinomycetes as effective biocontrol agent to improve crop yield for sustainable agricultural development.

K e y w o r d s

Actinomycetes, Antifungal activity, Biocontrol, Stress tolerance

Introduction

Fungi are a highly destructive pathogen. Many diseases and disorders can affect plants during the growing season. Fungal phytopathogens cause serious problems worldwide causing diseases like rusts, smuts, rots and wilt which damage the crops. Plant diseases need to be controlled to maintain the quality of food, feed, and fiber produced by growers around the world. Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products every year.

Fusarium oxysporium is highly destructive pathogen of both green house and field grown tomatoes in warm vegetable production areas, the disease caused by this fungus is characterised by wilting of plants and yellowing of leaves which reduces crop yield (Anitha and Rebeeth, 2009) *Fusarium soloni* which is responsible for root rot of tomatoes. *A. alternate* causes leaf spot and yellowing of leaves of tomatoes. *A. flavus* cause wilting of tomatoes. *C. gloeosporioides* is responsible for anthracnose in fruits and vegetables.

Pesticides are used to control plant diseases. However, agrochemical treatment causes environmental pollution and decreased diversity of non-target organisms. Microorganisms as biological control agents have high potential to control plant pathogens and no effect on the environment or other nontarget organism (Sutthinan Khamna, 2009).

There are numerous reports on the potential use of biocontrol agents as replacements for agrochemicals (Shimizu *et al.,* 2000). *Actinomycetes* are spore forming gram positive bacteria with high G + C content. They occur in the plant rhizosphere soil and produce active compounds (Suzuki *et al.,* 2000). Active *Actinomycetes* may be found in medicinal plant root rhizosphere soils and may have the ability to produce new inhibitory compounds against phytopathogens. Plant root exudates stimulate rhizosphere growth of streptomycetes that are strongly antagonistic to fungal pathogens. *Streptomyces sp*. Strain 5406 has been used in China to protect cotton crops against soil-borne pathogens. In the present study *Actinomycetes* were isolated from rhizosphere of various medicinal plants, and also soil near brick factory. All samples were collected from the Pune region in Maharashtra, India (18^o 32' 0" N, 73^o 52' 0" E.). The objective of this study was to further characterize these isolates for their enzymatic activities (chitinases, lipase, chitinases protease and β-1-3-glucanase) and physiological traits (temp, pH, salinity, growth in presence of different organic compounds). These isolates were screened for antiphytopathogenic activity against five selective plant pathogens.

Materials and Method

Sample collection and transport

Soil samples were collected from rhizosphere of medicinal plants such as Turmeric, Aloevera, Hibiscus and soil near brick factory from Pune region. All the samples were collected in sterilized autoclave bags, transferred to laboratory and maintained at 4^oC.

Sample pretreatment and isolation

Heat treatment was given to all the soil samples by keeping them in hot air oven at 50^oC for 1hr. The soil suspension was serially diluted and 0.1ml aliquots of each dilution was spread on CSA (casein starch agar) and AIA (*Actinomycetes* isolation agar) medium and incubated at 28^oC for 5-6 days. After incubation plates were observed for growth of *Actinomycetes* (Sakthi velayudham *et al.,* 2012).

Characterization of isolates

Colony morphology, Gram's staining and slide culture assembly was performed (Brenner *et al.,* 2005). Biochemical tests were performed with reference to Bergey's manual of determinative bacteriology.

Phenotypic characterizations

a) Aerial mass colour

For the grouping and identification of *Actinomycetes sp*. the chromogenicity of the

aerial mycelium is considered to be an important character. The colors of the mature sporulating aerial mycelium are white, gray, red, green, blue and violet. When the aerial mass colour falls between two colors series, both the colors are recorded. In the cases where aerial mass color of a strain showed intermediate tints, then in that place both the colour series should be noted (Shirling and Gottileb, 1966).

b) Reverse side pigments

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive or none (-).

A colour with low chroma such as pale yellow, olive or yellowish brown occurs, it is included in the latter group (-).

Determination of enzymatic activity

Chitin degradation: Chitinase activity of the isolates was determined on colloidal chitin agar plates containing swollen chitin 2g L^{-1}, ammonium sulphate 0.05gL^{-1}, agar 1.8g L^{-1} and pH was adjusted at 7. The isolates were spot inoculated and Chitinase activity was identified by clear zone around the colony after 5days of incubation at 28^{o}C (Sasikumar Arunachalam Palaniyandi *et al.,* 2013).

Cellulose degradation: Cellulose degradation was determined by spot inoculating *Actinomycetes* on Czepak mineral salt medium and incubated at 28^{o}C for 2-3 days. For detection of cellulose degradation plates were flooded with 1% Congo red solution. After 5 minutes excess dye was drained. 1M NaCl was then added repeatedly until color disappeared. Clear zone of hydrolysis around the cellulase-producing organism was observed due to hydrolysis of cellulose.

Hydrolysis of casein: The protease activity was determined in a medium containing 10g L^{-1} casein, 10g L^{-1} glucose, 1.5g L^{-1} K_2HPO_4 and 15g L^{-1} agar (Sasikumar Arunachalam Palaniyandi *et al.,* 2013). The isolates were spot inoculated on the medium and incubated at 28^{o}C for 5 days, proteolytic activity was identified by a clear zone around the colony.

Hydrolysis of starch: Amylase activity of the isolates was determined on starch agar plate. The isolates were spot inoculated and incubated at 28^{o}C for 3-5 days; amylase activity was identified by flooding the plates with 1% iodine and observing clear zone around the colony.

Hydrolysis of gelatin: Gelatin hydrolysis was observed by spot inoculating the nutrient agar plates containing 15% gelatin, after incubation plates were flooded with 1% $HgCl_2$ and clearance was observed around the growth.

Collection of fungal cultures

Fungal cultures for the study such as *F. oxysporium, F. solani, A. alternata, A. flavus, C. gloeosporioides* were procured from Agharkar Research Institute, Pune.

***In vitro* antifungal assays**

Agar streak method

The antiphytopathogenic activity of all isolates was analyzed by agar streak method (Dhanasekaran *et al.,* 2011). Each of the isolates was streaked as a straight line on casein starch agar plates and incubated at 28^{o}C for 6 days. After the 6th day, different fungal pathogens were streaked at right angle, but not touching each other and then

incubated at 28^{o}C for 2-3 days. If the organism is susceptible to the antibiotic produced by actinobacteria then it will not grow near the actinobacteria. The zone of inhibition against each test fungal pathogen was noted (Dhanasekaran *et al.,* 2011).

Dual culture method

One 5mm^2 disk of pure fungal culture was placed at the centre of petridish containing PDA. A circular line, made with a 4cm diameter petridish dipped in a bacterial suspension of 2*10^9 cfu/ml was placed surrounding the fungal inoculum. Plates were incubated at 28^{o}C for 72h and growth diameter of the pathogen (fungal growth) was measured and compared with control, where the bacterial suspension was replaced with LB broth (Mohsin Tariq *et al.,* 2010). Percent inhibition was calculated using the following formula

% inhibition = [1-(treatment growth / control growth * 100)

Stress tolerance

The effect of temperature, alkalinity, salinity and calcium salts was studied on *Actinomycetes* isolates by observing their growth on casein starch under different stress parameters. The effect of temperature was studied by incubating the isolates at 15^{o}C, 30^{o}C and 45^{o}C. The influence of alkalinity on *Actinomycetes* growth was studied by growing the isolates at pH 7, pH 8, pH 9 and pH 10. The *Actinomycetes* were grown on agar media with different NaCl (2.5%, 5%, 7.5% and 10%), $CaCO_3$ (2.5% and 5%), $CaSO_4$ (2.5% and 5%) and $CaCl_2$ (2.5% and 5%).

Molecular identification of potential isolates: 16s rDNA partial gene sequencing was done by isolating and purifying the genomic DNA of potential isolates. This work was carried out in association with Genombio technologies, Pune. The nucleotide sequence obtained were compared using the BLASTN programme on the page of National centre for biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and BLAST analysis was realized.

Result and Discussion

Total ten bacterial isolates were isolated from the four different soil samples. All the isolates were designated as shown in Table no. 1 The isolated strains were filamentous, Gram positive, non motile and aerobic in nature, having Catalase and Oxidase activities. Also slide culture observation indicates that isolates belonged to genus *Streptomyces*. All tested actinobacterial cultures utilized every tested rhizospheric sugar indicating higher probability of rhizosphere survival. Results of aerial mass colour, reverse side pigments, biochemical characterization, enzymatic activity, antifungal activity against selected phytopathogens and stress tolerance on growth, such as salt tolerance, growth at different pH, growth in presence of $CaCO_3$ and $CaSO_4$ of *Actinomycetes* were given in the tables 2 to 9. All isolates were able to grow at 15^0 to 55^{0}C and unable to grow in presence of 2.5 and 5 % $CaCl_2$

Actinomycetes isolate A3 with good antiphytopathogenic activity against all phytopathogens tested and significant activity against *C.gloeosporioides which* is 86.66%. BF5 showing percent inhibition more than 50 for four fungal pathogens and significant activity against *F.solani* (70.49%).

Table.1 Isolation of *Actinomycetes* from various areas

S.No	Location	Isolates
1	Aloevera rhizosphere	A1
2		A3
3	Hibiscus rhizosphere soil	H2
4		H4
5		H5
6		H6
7	Brick factory soil	BF2
8		BF3
9		BF5
10	Turmeric rhizosphere soil	Tu

Table.2 Phenotypic and biochemical characterization of *Actinomycetes* isolates

S.No	Isolates	Phenotypic and Biochemical characterization							
		Substrate mycelium	Aerial mycelium	Aerial mass colour	Reverse side colour	H_2S	Urease	Catalase	Oxidase
1	A1	+	+	Grey	Yellowish	+	+	+	+
2	A3	+	+	Grey	Yellowish orange	-	+	+	+
3	H2	+	+	White	Yellowish white	+	+	+	+
4	H4	+	+	Greenish grey	Yellowish green	+	+	+	+
5	H5	+	+	Grey	Grey	+	+	+	+
6	H6	+	+	Off-white	greenish	+	+	+	+
7	BF2	+	+	Grey	Orange	+	+	+	+
8	BF3	+	+	Black	Light orange	+	+	+	+
9	BF5	+	+	White	Pale yellow	+	+	+	+
10	Tu	+	+	White	Redish brown	-	+	+	+

Table.3 Enzymatic activity of *Actinomycetes* isolates

S. No	Isolates	Enzymatic activity					
		Chitinase	Glucanase	Amylase	Caseinase	Cellulase	Gelatinase
1	A1	+	+	+	+	+	+
2	A3	+	+	+	+	+	+
3	H2	+	+	+	-	+	+
4	H4	-	+	+	+	+	+
5	H5	+	+	+	-	+	+
6	H6	+	+	+	+	+	+
7	BF2	+	+	+	+	+	+
8	BF3	+	+	-	+	+	+
9	BF5	+	+	+	+	+	+
10	Tu	+	+	+	+	+	+

+: positive -: negative

Table.4 Screening of Antifungal activity of *Actinomycetes* isolates agar streak method

S. No	Isolates	*F. oxysporum*	*F. solonae*	*A. alternate*	*A. flavus*	*C. gleosporoids*
1	A1	+	+	+	+	-
2	A3	+	+	+	+	+
3	H2	+	-	+	-	+
4	H4	+	+	-	+	-
5	H5	+	+	-	+	-
6	H6	+	+	+	-	+
7	BF2	+	+	+	-	+
8	BF3	+	-	+	-	+
9	BF5	+	+	+	-	+
10	Tu	+	+	+	+	-

+: positive -: negative

Table.5 Percent inhibition of antifungal activity by Dual culture assay

S. No	Isolates	*F. oxysporium*	*F. solani*	*A. alternata*	*A. flavus*	*C. gloeosporioides*
1	A1	51.42	42.85	34.42	71.42	-
2	A3	57.14	71.42	39.34	45.71	86.66
3	H2	57.14	-	50.81	-	60.00
4	H4	48.57	47.14	-	44.28	-
5	H5	54.28	57.14	-	55.71	-
6	H6	28.57	42.85	49.18	-	46.66
7	BF2	54.28	42.85	45.90	-	70.00
8	BF3	62.85	-	55.73	-	80.00
9	BF5	54.28	65.71	70.49	-	53.33
10	Tu	64.28	50.00	34.42	65.71	-

-: no inhibition

Table.6 Sodium chloride tolerance on the growth of *Actinomycetes* isolates

S. No	Isolates	2.5%	5%	7.5%	10%
1	A1	+	+	+	-
2	A3	+	+	-	-
3	H2	+	+	+	-
4	H4	-	-	-	-
5	H5	+	-	-	-
6	H6	+	+	-	-
7	BF2	+	+	+	-
8	BF3	+	+	+	-
9	BF5	+	+	+	-
10	Tu	+	+	+	-

+: positive -: negative

Table.7 Effect of different pH on the growth of *Actinomycetes* isolates

S. No	Isolates	pH 7	pH 8	pH 9	pH 10
1	A1	++++	+++	+++	+
2	A3	++++	++	+	+
3	H2	+++	++	+	+
4	H4	+++	+++	+++	+
5	H5	+++	+++	+++	+
6	H6	+++	+++	+++	+
7	BF2	+++	+++	+++	+
8	BF3	++++	+++	+++	+
9	BF5	++++	+++	+++	+
10	Tu	+++	+++	+++	++

+ Poor growth, ++ moderate growth, +++good growth, ++++ luxuriant growth

Table.8 Effect of different concentration of calcium carbonate on the growth of *Actinomycetes* isolates

S. No	Isolates	$CaCO_3$ concentration (%)	
		2.5	5
1	A1	+	+
2	A3	+	+
3	H2	+	+
4	H4	+	+
5	H5	+	+
6	H6	+	+
7	BF2	+	+
8	BF3	+	+
9	BF5	+	+
10	Tu	+	+

+: positive -: negative

Table.9 Effect of different concentration of calcium sulfate on the growth of *Actinomycetes* isolates

S. No	Isolates	$CaSO_4$ concentration (%)	
		2.5	5
1	A1	+	+
2	A3	+	+
3	H2	+	+
4	H4	+	-
5	H5	+	+
6	H6	+	+
7	BF2	+	+
8	BF3	+	+
9	BF5	+	+
10	Tu	+	+

+: positive -: negative

The molecular identification by 16SrDNA partial sequencing and morphological characteristics confirmed that isolates A3 and BF5 are confirmed as *Streptomyces sp*p. Thangapandian *et al.* (2007) isolated Streptomyces from medicinal plant rhizosphere soils and 8 isolates had antipathogenic activity. Crawford *et al.* (1993) found that 12 actinomycete strains isolated from Taraxicum officinale rhizosphere were active against *Pythium ultimum.* Sutthinan Khamna *et al.* (2009) isolated *Actinomycetes* from rhizosphere of medicinal plants and demonstrated the antiphytopathogenic activity of streptomycetes against selective plant pathogen. Kanini *et al.* (2013) isolated and identified potential antifungal streptomycetes from rhizosphere and nonrhizosphere soil and carried out in vivo experiments on beans. Srividya *et al.* (2012) evaluated *Streptomyces sp*. 9p as effective biocontrol against chilli soil borne fungal phytopathogens

Streptomyces spp. reported in this study shows significant antagonistic activities against some important phytopathogens. The pH of soil in Maharashtra is high in most districts except Konkan area (survey report Indian institute of soil science, Bhopal) Stress tolerance studies have shown that these isolates are showing good growth at higher pH, temperature, salt concentration. Further studies on antifungal compounds from this isolate and in vivo experiment should be carried out to use these organisms as biocontrol agents. This work leads and support use of *Actinomycetes* as effective biocontrol agent to improve crop yield for sustainable agricultural development.

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References

Anitha, A., Rebeeth, M. 2009. In vitro Antifungal activity of *Streptomyces griseus* against phytopathogenic fungi of tomato field. *Aca. J. Plant Sci.*, 2(2): 119–123.

Brenner, D.J., Krieg, N., Staley, J.R. 2005. Bergey's manual of systematic bacteriology, Vol. 2. Springer, New York.

Crawford, D.L., *et al.* 1993. Isolation and characterization of *Actinomycete* antagonists of a fungal root pathogen. *Appl. Environ. Microbiol.*, 59(11).

Dhanasekaran, D., Thajuddin, N., Pannnerselvam, A. 2011. Applications of actinobacterial fungicides in agriculture and medicine. Fungicides for plant and animal diseases. In Tech Open Access publisher. Pp. 29–54.

Kanini, G.S., Katsifas, E.A., Savvides, A.L., Hatzinikolaou, D.G., Karagouni, A.D. 2013. Greek indigenous streptomycetes as biocontrol agents against the soil-borne fungal plant pathogen *Rhizoctonia solani. J. Appl. Microbiol.*,

Mohsin Tariq, Sumera Yasmin, Fauzia Y. Hafeez, 2010. Biological control of potato black scurf by rhizosphere associated bacteria. *Braz. J. Microbiol.*, 41: 439–451.

Sakthi Velayudham, Kasi Murugan, 2012. Diversity and antibacterial screening of *Actinomycetes* from Javadi Hill Forest soil, Tamilnadu, *India. J. Microbiol. Res.*, 2(2): 41–46.

Sasikumar Arunachalam Palaniyandi, Seung Hwan Yang, Karthiyaini Bamodharan, Joo-Won Suh, 2013. Genetic and

functional characterization of culturable plant-beneficial actinobacteria associated with yam rhizosphere. *J. Basic Microbiol.*, Pp. 1–11.

Shimizu, M., Yazawa, S., Ushijima, Y. 2009. A promising strain of endophytic *Streptomyces sp.* For biological control of cucumber anthracnose. *J. Gen. Plant Pathol.*, 75: 27–36.

Shirling, E.B., Gottlieb, D. 1966. Methods for characterization of *Streptomyces sp*ecies. *Int. J. Syst. Bacteriol.*, 16(3): 313–340.

Srividya, S., Adarshana Thapa, Deepika V. Bhat, Kajingailu Golmei, Nilanjan Dey, 2012. Streptomycessp. 9p as effective biocontrol against chilli soilborne fungal phytopathogens. *Eur. J. Exp. Biol.*, 2(1): 163–173.

Sutthinan Khamna, Akira Yokota, John F. Peberdy, Saisamorn Lumyong, 2009. Antifungal activity of *Streptomyces sp*p. isolated from rhizosphere of Thai medicinal plants. *Int. J. Integrative Biol.*, 6(3): 143.

Suzuki, S., Yamamoto, K., Okuda, T., Nishio, M., Nakanishi, N., Komatsubara, S. 2000. Selective isolation and distribution of *Actinomadura rugatobispora*. strains in soil. *Actinomycetologica*, 14: 27–33.

Thangapandian, V., Ponmuragan, P., Ponmuragan, K. 2007. *Actinomycetes* diversity in the rhizosphere soil of different medicinal plants in Kolly Hills, Tamilnadu, India, for secondary metabolite production. *Asian J. Plant Sci.*, 6: 66–70.