JOURNAL OF MODERN BIOTECHNOLOGY, VOL. 2, NO. 3, pp 66–72, May 2013

ISSN 2319–6645
JOURNAL OF MODERN BIOTECHNOLOGY

www.thebiotech.org

Research Article

Antagonistic Activity of Actinomycetes from Jeypore Paddy Soils against Selective Phytopathogenic Fungi

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Received 10 March 2013; Revised 05 April 2013; Accepted 17 April 2013

Abstract

In the present study, 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 orysporum* by dual plate assay. The antagonistic effects were more prominent after two days in *R. solani* and three days in the other fungi. Act 8 and 10 resulted in an inhibition of 1.1–1.5 cm, whereas others showed a minimum inhibition of <0.8 cm. The colony morphology of actinomycetes was observed under phase contrast microscope and it revealed the highly coiled spiral type of spore chains particularly in Act 7, 9 and 11. Genomic DNA was isolated from the selected isolates and they were amplified using PCR primers fD1 and rP2, which produced amplification of 1500 bp. RFLP was used to analyze the genetic diversity of the actinomycetes isolates from the soil using restriction enzyme Sau3A, which formed restriction pattern producing bands with 540–650bp confirming the isolates were belongs to the genes *Streptomyces*.

Keywords: Pathogens, dual plate, antagonistic activity, RFLP, PCR amplification, *Streptomyces*

INTRODUCTION

Actinomycetes are among the most widely distributed group of microorganisms in nature (Oskay *et al.*, 2004). They are found abundantly in cultivated and uncultivated soils in various regions throughout the world (Goodfellow and Simpson, 1987). Although soils have been screened by the pharmaceutical industry for about 50 years, only a miniscule fraction of the surface of the globe has been sampled and only a small fraction of actinomycetes taxa has been discovered (Baltz, 2007). Actinomycetes are filamentous, gram-positive bacteria producing antibiotics of agricultural and medicinal importance. Morphological characters are still widely used for characterizing genera, the presence or absence of spores on the substrate mycelium or the formation of zoospores in specialized spore vesicles or sporangia. The ability to produce motile spores is more widespread in the actinomycetes. Besides the enormous numbers of agroactive metabolites produced by actinomycetes (Tanaka and Omura, 1993), they also play an important role in agriculture as biocontrol agents. Antagonism against an extensive variety of plant pathogens has been reported (Chamberlain and Crawford, 1999; Doumbou *et al*., 2002). The antagonistic activity of actinomycetes to fungal pathogens is usually related to the production of antifungal compounds (Ouhdouch *et al*., 2001; Getha and Vikineswary, 2002). Each actinomycete strain has probably genetic potential for producing 10–20 secondary metabolites (Bentley, 2002). Among, many species of actinomycetes, particularly, those belonging to the genus *Streptomyces* produces and secrete a wide array of biologically active compounds including antibiotics, hydrolytic enzymes and enzyme inhibitors (Compant *et al*., 2005; Fravel *et al*., 2005; Shantikumar *et al*., 2006) enhances soil fertility and have been proved to possess antagonistic activity against wide range of soil-borne plant pathogens (Aghighi *et al*., 2004).

Therefore, in this study an effort was made to screen actinomycetes for *in vitro* inhibition activity against phytopathogenic fungi using strains isolated from paddy soil samples in Jeypore, Odissa.

MATERIALS AND METHODS

Soil sample collections

Soil samples were collected from the upland paddy field Jeypore, Odissa and were transported aseptically in sterile plastic containers to the laboratory. The samples were dried overnight. The texture of the samples varied from sandy to loamy which were identified by feel method. pH and EC were analyzed in soil sample.

Isolation of actinomycetes

One gram of soil sample was serially diluted up to 10^{-7} dilution. Aliquots of 0.1 mL of each dilution was spread plated on starch casein agar plates in triplicates and incubated at room temperature for 7 days. After the incubation period, the plates were examined for the presence of actinomycetes colony. The suspected colonies were picked up and purified on International Streptomyces Project (ISP-2) agar by Waksman (1961) media and incubated at room temperature for about 4 days. The suspected pure actinomycetes culture was inoculated on ISP-2 slants; after the incubation period, the slants were taken for further identification and antifungal screening. The stock culture was preserved in 15% glycerol (v/v) at −20°C (Maniatis, 1989).

Culture characteristics

Pure isolates of actinomycetes were used to study the morphological characteristics such as substrate and aerial mycelia of spore bearing hyphae with the entire spore chain and structure of the spore chain with the actinomycetes morphologies. This was done by using cover slip method in which individual cultures were transferred to the base of cover slips buried in ISP-2 medium (Williams and Wilkins, 1994).

Test organism

The plant pathogens that cause economic loss to paddy namely *Rhizoctonia solani, Helminthosporium oryzae, Fusarium oxysporum and Curvularia lunata* were selected for the study. The pure cultures were obtained from the Biocontrol and Microbial Metabolites Lab, Center for Advanced Studies in Botany, University of Madras, Chennai.

Antifungal activity of actinomycetes

In vitro antagonism test, using dual culture method was used to examine the inhibition of phytopathogenic fungi, *R. solani*, *H. oryzae*, *C. lunata* and *F. orysporum*. Test media (ISP-2) for the dual culture method were selected based on the growth performances of both test actinomycetes and fungi. Size of the culture plates was determined based on the growth rates of fungi on test medium (90 mm diameter plates for all). Petri plates were inoculated with actinomycetes 24 to 48 h prior to the fungal inoculation. Spores of the 15 actinomycetes were uniformly spotted equidistantly near the periphery of the each plate and when these strains were visibly growing, 5-mm diameter agar plugs taken from a growing edge of a 5-day-old test fungal colony was transferred to the centre of the test agar plate surface. Cultures were incubated at room temperature (30±2°C) and when growing edges of control fungi (without any actinomycete inoculum) were at the edge of the plates, the diameters of the test fungal colonies toward each actinomycete were measured.

Genomic DNA extraction

Actinomycete strains were grown in 10 mL of ISP-1 (Shirling and Gottlieb, 1966) with agitation at 30°C for 72 h. Cells (2 mL) were harvested by centrifugation (7500 rpm for 10 min), suspended in 500 µl STE buffer (pH 8.5) and homogenized with micro-tip. To this 200 µl (80 mg/mL) lysozyme was added and kept for overnight incubation at room temperature. This was suspended in 250 µl SDS (10%), 250 µl of 3.5M sodium acetate, kept on vortex for 1min and centrifuged at 12,000 rpm for 10 min. To the supernatant, equal volume of absolute alcohol was added, incubated at –20°C for 2h and centrifuged (12,000 rpm for 15min). The pellet was suspended in 70% ethanol, centrifuged twice or thrice (12,000 rpm for 10 min) and kept for complete alcohol evaporation (2–4h). The DNA sample obtained was diluted to 80 µl. The genomic DNA was checked for its purity and stored in –70°C for further use.

PCR amplification and RFLP

PCR (Hot Bonnet, Minicycler™) was carried out in 20 µl volume containing 2 µl of 10X buffer (1.5 mM *Taq* buffer, $MgCl_2$), 3 U *Taq* polymerase, 2 mM of dNTPs, 25 ng of each primer and 2 µl template DNA. Primer fD1 (5'-AGTTTGATCCTGGCTCAG-3') and primer rP2 (5'-ACGGCTACCTTGTTACGACTT-3') were used. Primer fD1 binds to base positions 26–43 and primer rP2

to base positions 1494–1474 of the 16S rRNA gene of *Escherichia coli*. The primers were used to amplify nearly full-length 16S rRNA sequences. The PCR programme used was as follows: initial denaturation (94°C for 4 min), 30 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min), extension (72°C for 1 min), a final extension (72°C for 1 min) and incubation (16°C for 24 h). The PCR products were electrophoresed on 2% agarose gels, containing 1 µl ethidium bromide (10 $\mu g/mL^{-1}$), to ensure that a fragment of the correct size had been amplified.

Master mix of the enzyme (0.5 µl/sample) and buffer (3 µl/sample) was prepared with the following composition. 7 µl of the amplified DNA sample was taken and the master mix was added to it. The samples restricted with *Sau*3AI were incubated at 37°C for 1 h. Then the samples were loaded in 2% agarose gel to visualize the restricted banding patterns using UV transilluminator. Finally a phylogenetic tree was constructed using MEGA 3.1 cluster analysis.

RESULTS AND DISCUSSION

Soil samples were collected from the upland paddy field Jeypore, Odissa. The pH of the soil samples were found as acidic ranging from 4.5 to 5.7 and EC analysis showed the EC levels as normal ranging from 0.04 to 0.254 $m.mho^{-1}cm$. The ideal pH range for soil is from 6.0 to 6.5 because most plant nutrients are in their most available state. Soil pH may also influence nutrient availability and microbial activity in general and thereby have a direct effect on disease suppression (Simon and Sivasithambaram, 1990; Ownley *et al*., 1992).

One gram of soil sample was dried and taken for isolation of actinomycetes. 15 actinomycetes isolates were isolated and inoculated on the ISP-2 medium. The isolated cultures were designated as Act-1 to 15. The isolates were aerobic and Gram positive. Slide cultures showed that it had aerial mycelia with sporangium. Majority of the isolates were white to grey in color (Table 1). Spore chain arrangements observed using phase contrast microscope at higher magnification showed that all the isolates bear spore chains of 3 or more and are non motile. It was observed that isolate Act-7, 9 and 11 form a spore chains like structure which can only be found in the *Streptomyces* sp. (Figure 1). Actinomycetes (*Streptomyces* sp.) isolated from rhizosphere soil have been reported to produce siderophores and inhibit the growth of phytopathogens (Tokala *et al*., 2002).

All the 15 cultures were screened against phytopathogenic fungi (Table 2). Among the 15 isolates of actinomycetes, only 8 isolates showed activity against the phytopathogens. Biological control agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived level of safety and minimal environmental impacts (Pal and Gardener, 2006). Act-8 and Act-10 showed very good activity against all the pathogens. Also Act-7 and Act-15 showed good antagonistic activity against *H. oryzae* and *C. lunata* (Figure 2). Previous reports also suggest that actinomycetes isolated from Malaysia soil had the potential to inhibit the growth of several tested plant pathogens (Jeffrey *et al*., 2007). Aghighi *et al*. (2004) have shown that from 110 isolates only 14 isolates were found to be active against *Alternaria solani*, *A. alternata*, *Fusarium solani*, *Phytophthora megasperma*, *Verticillium dahlia* and *Saccharomyces cerevisiae*. Bonjar *et al*. (2005) assayed antifungal actinomycetes strains for antagonistic activity against *V. dahlia*, *A. solani*, *F. solani* and *Geotrichum candidum* four worldwide phytopathogenic fungi. Prapagdee *et al*. (2008) reported that of the 146 strains of indigenous *Streptomyces* isolated from rhizosphere soils (paddy and orchards fields), only 10 strains exhibited antifungal activity. Khamna *et al*. (2009) isolated 396 *Streptomyces* strains from 16 rhizosphere soil samples (medicinal plants). Bharti *et al*. (2010) obtained 316 *Streptomyces* strains from different soil samples, of which, 31% exhibited antifungal activity. Antibiotic producers were readily isolated from soils that are naturally suppressive to diseases such as take-all of wheat (Weller *et al*., 2002), black rot of tobacco (Keel *et al*., 1996) and Fusarium wilt of tomato (Tamietti *et al*., 1993), indicating that they may play an important role in the natural biological control that occurs in these soils. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Errakhi *et al*., 2007; Khamna *et al*., 2009). The mechanism of antifungal antagonists can be due to the secretion of hydrolytic enzymes such as chitinase, b,3 glucanase, chitosanase, and proteases (Yuan, 1995) which degrade the fungal cell wall or the secretion of antifungal compounds (Khamna *et al*., 2009).

Phylogenetic analysis of 16S rRNA technique of the 15 isolates of actinomycetes showed that all the isolates belong to the genus *Streptomyces* but of different species. RFLP using *Sau*3AI was used to analyze the genetic diversity of the actinomycete isolates from the soil samples of Jeypore (Figure 3). According to the enzyme restricted, the 16S rDNA fragment pattern showed no notable differences within the actinomycete isolates and was found to belong to the genus *Streptomyces* sp. The most important restriction endonuclease used in this rapid identification method is *Sau*3AI. The dendrogram pattern of the 15 actinomycete isolates formed two main clusters, of which one clusters consist of only one isolate (Act-15) and remaining forms two sub clusters and further formed many subcultures (Figure 4). A rapid method to distinguish *Streptomyces* spp. from other actinomycetes and to identify the non-streptomycetes to the genus level would be extremely useful (Cook and Meyers, 2003). *Streptomyces* sp. has been shown to protect many crop plants against pathogenic fungi (Liu *et al*., 1996). The present work has resulted in selective isolation of novel soil *Streptomyces* spp. and their antifungal activity against some plant pathogenic fungi. But more precise work and further development in this field is required to produce more potent bioactive antifungal compounds from *Streptomyces* spp. which are easily available in the soil.

Table 1: Culture characteristic of the actinomycetes in ISP-2 medium

Isolate Code	Colony Color	Pigmentation
Act-1	White to Grey	–
Act-2	White to Grey	–
Act-3	Dirty White	–
Act-4	White	–
Act-5	Light blue	Brown
Act-6	White	–
Act-7	White	–
Act-8	White to Grey	–
Act-9	Grey	–
Act-10	Dirty White	–
Act-11	Brown to grey	–
Act-12	Grey	–
Act-13	White	Yellow
Act-14	White	–
Act-15	Dirty White	Brown

–: No pigmentation

Table 2: Antagonistic activity of actinomycetes isolates against fungal pathogens

Isolate Code	*R. solani*	*H. oryzae*	*C. lunata*	*F. oxysporum*
Act-1	–	–	–	–
Act-2	–	–	–	–
Act-3	–	–	w	w
Act-4	–	–	–	–
Act-5	–	–	–	–
Act-6	–	–	–	–
Act-7	w	1.5	1.4	w
Act-8	1.1	1.1	1.5	w
Act-9	–	–	w	–
Act-10	1.3	1.5	1.4	1.5
Act-11	–	–	–	–
Act-12	w	–	–	–
Act-13	w	–	–	–
Act-14	–	w	w	w
Act-15	w	1	1.1	w

–: No activity; w: less activity

ACKNOWLEDGEMENT

Authors are thankful to Prof. M. S. Swaminathan Research Foundation, III Cross Street, Taramani Institutional Area, Chennai 600 113, Tamil Nadu, India for providing the necessary facility to carry out the work.

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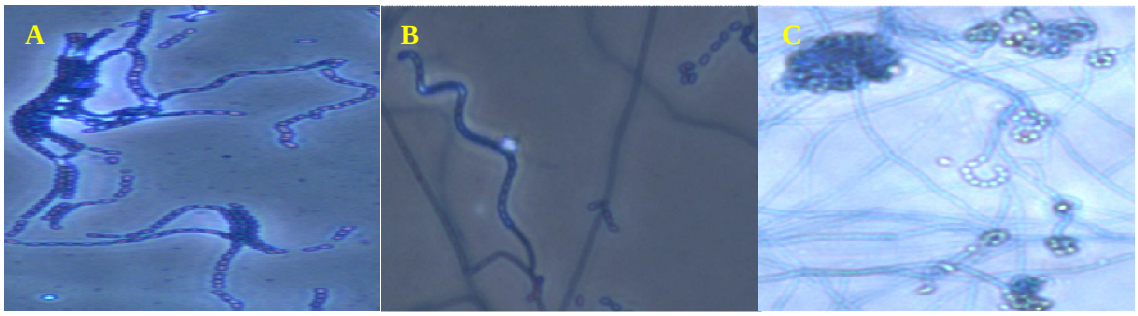

Figure1: Spore chain arrangement observed at 1000X using Phase contrast microscope.

A: Chained spore of Act-7; B: Highly coiled spore of Act-9; C: Chained spore of Act-11

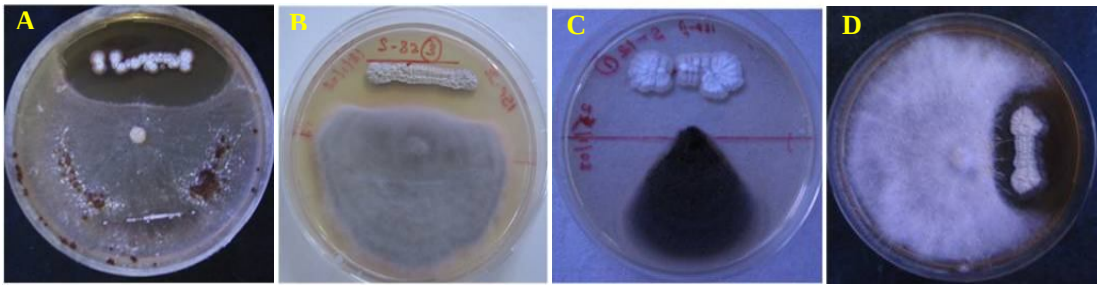

Figure 2: Antagonistic activity of actinomycetes against phytopathogens.

A: *R. solani*; B: *H. oryzae*; C: *C. lunata* and D: *F. oxysporum*

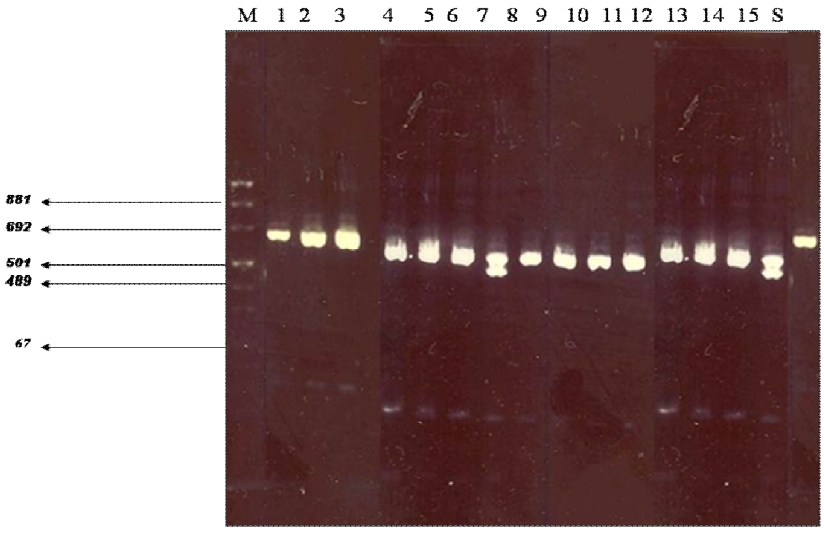

Figure 3: Agarose gel electrophoresis of RFLP with the standard Streptomyces sp. Culture.

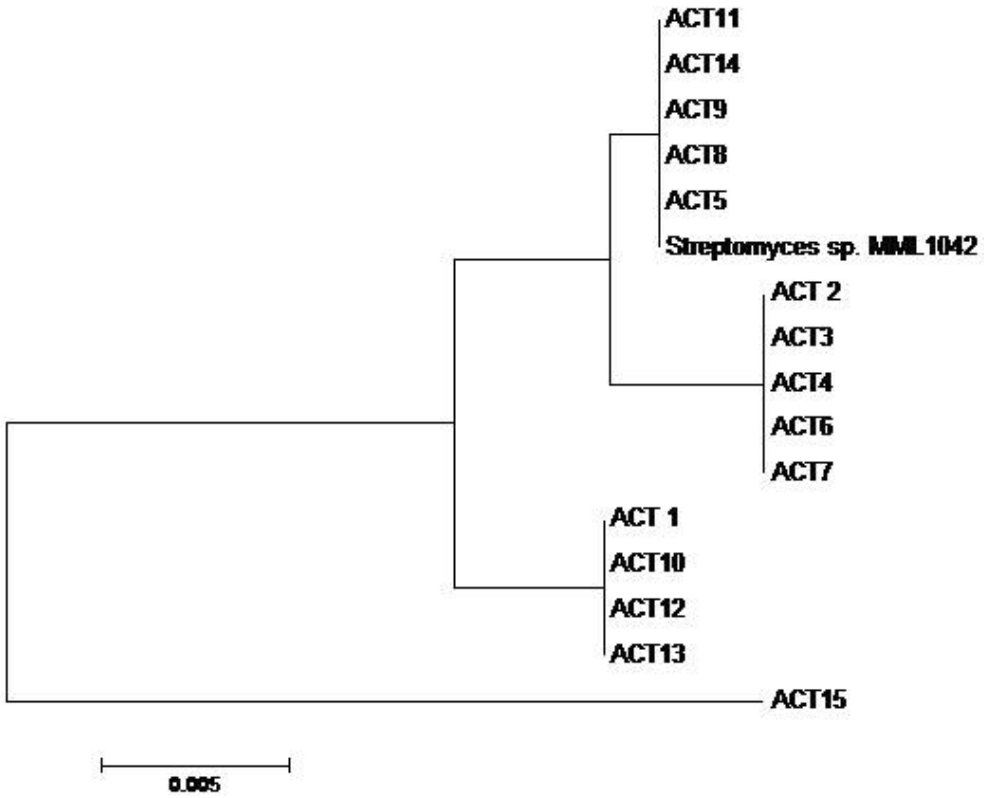

Figure 4: RFLP pattern of amplified 16S rDNA genes from actinomycetes restricted with *Sau3A1*

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