

Original Research Article

Actinomycetes from *Capsicum annuum* L. Rhizosphere Soil Have the Biocontrol Potential against Pathogenic Fungi

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ABSTRACT

Keywords

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A total of 54 actinomycetes strains were isolated from *Capsicum annuum* L. rhizosphere soils of Warangal district, Andhra Pradesh, India. They were screened for their antifungal activity against *Colletotrichum capsici* and *Fusarium oxysporum*. Out of 54 strains, 25 showed antifungal activity against *Colletotrichum capsici* and from among these 25 strains 13 were antagonistic to *Fusarium oxysporum*. All antifungal strains were Gram positive and produced different pigments. These antifungal strains exhibited a diversity of biochemical characteristics viz., amylase, chitinase, cellulase, pectinase, protease, catalase, lipase and gelatin hydrolysis. Four strains OUA3, OUA5, OUA18, and OUA40 were positive for all the enzymes and the strain OUA36 failed to show any enzyme activity. Majority of antifungal actinomycetes (76%) produced chitinases and other hydrolytic enzymes emphasizing their utility as biocontrol agents against phytopathogenic fungi.

Introduction

Actinomycetes are Gram positive, filamentous, sporulating bacteria with DNA rich in G+C content ranging from 55-75% (Ho *et al.*, 2002). These are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), antibiotics (Strohl, 2004), antitumor agents (Cragg *et al.*, 2005), immunosuppressive agents (Mann, 2001) and enzymes (Oldfield *et al.*, 1998). Actinomycetes are unparalleled source of not only bioactive metabolites

including antibiotics, plant growth factors and other substances (Shahidi *et al.*, 2004), they are effective biocontrol agents against many phytopathogenic fungi (Crawford *et al.*, 1993).

Fungal pathogens pose serious problem world wide and cause a number of plant diseases including rusts, smuts, rots, wilt, anthracnose causing severe damage to crops. *Colletotrichum*, the anthracnose pathogen of chillies affect the crop yield

up to 50% (Pakdevaraporn *et al.*, 2005) while *Fusarium oxysporum* about 20%. Actinomycetes inhabit a diversity of habitats from aquatic, marine, mangrove to terrestrial environments (Mohan and Singara Charya, 2012; Gulve and Deshmukh 2011, Gomes *et al.*, 2000). Soil actinomycetes particularly *Streptomyces* sp. enhance soil fertility and have antagonistic activity against wide range of soil borne plant pathogens (Aghighi *et al.*, 2004). Biological control of phytopathogenic fungi by chitinolytic microorganisms is a proven concept and the role of cell wall degrading enzymes as the mechanism involved in the management of fungal pathogens has been suggested by Elad *et al.*, (1982). The aim of this study was to explore the rhizosphere soils of chilli (*Capsicum annuum* L.) for potential actinomycete strains with the ability to produce hydrolytic enzymes like chitinases for their possible use as biocontrol agents against pathogenic fungi of chillies and other crops.

Materials and Methods

Collection of soil samples

Rhizosphere soil samples of *Capsicum* plants were collected from the agricultural fields located at various mandals viz., Sangem, Nekkonda, Chennaraopet, Narsampet, Parkal, Regonda, Mulugu of Warangal district, Andhra Pradesh, India. The samples were taken from the growing roots up to a depth of 5 cm after removing approximately 3cm of the soil surface. These samples were placed in polythene bags, closed tightly and analysed for actinomycetes.

Isolation of actinomycetes

The actinomycetes were isolated by serial

dilution method (Lingappa and Lockwood, 1961) on chitin medium (Aneja, 2003) and starch casein agar medium (Williams and Davies, 1965). 1ml aliquots were added to cool and solidified agar medium. The plates were incubated at $28\pm 2^\circ\text{C}$ for 8 days and the slow growing typical actinomycete colonies were subcultured.

Isolation of test pathogens

The test pathogens used in present study were *Colletotrichum capsici* and *Fusarium oxysporum* isolated from the diseased chilli fruits and plants respectively. The pathogens were single spored and tested for pathogenicity and stored at 4°C for further use.

Screening for antifungal activity

The actinomycetes strains were screened for antifungal activity *in vitro* against *C. capsici* and *F. oxysporum* by dual culture plate technique (Upadhyay and Rai, 1987). The percentage inhibition of mycelial growth of the pathogen was calculated by measuring the radial growth of the fungal strains in dual culture plate with that in control plate after seven days of incubation at 28°C . They were observed for zone of inhibition, contact inhibition and over growth. The percentage inhibition of mycelial growth of the pathogen was calculated using the formula given by Idris *et al.*, (2007).

Percent inhibition = $(R - r) / R \times 100$;
Where 'r' is the radial growth of the fungal colony (mm) opposite to the antagonistic strain, R is the radial growth of pathogen (mm) in control.

In vitro assay for biochemical characteristics

The strains tested positive for antifungal

activity (OUA3, OUA5, OUA7, OUA8, OUA9, OUA12, OUA14, OUA15, OUA16, OUA17, OUA18, OUA27, OUA28, OUA29, OUA30, OUA31, OUA32, OUA33, OUA36, OUA37, OUA38, OUA39, OUA40, OUA41 and OUA50) were assayed for biochemical characteristics.

Amylase production

Actinomycetes strains were streaked on starch agar (Beef extract 3.0, Peptone 5.0, Soluble Starch 2.0, Agar 15.0, Distilled water 1000ml) medium plates and incubated at 30°C for 48 h. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form a blue color compound. This blue color fades rapidly. Hence the colorless zone surrounding colonies indicates the production of amylase.

Chitinase production

The qualitative assay for chitinase production was performed on colloidal chitin medium (Colloidal chitin 4.0, K₂HPO₄ 0.7, KH₂PO₄ 0.3, MgSO₄ 0.5, FeSO₄ 0.01, ZnSO₄ 0.001, MnCl₂ 0.001, Agar 15.0, Distilled water 1000 ml) for 4 days at 28±2°C. Clear halos around and beneath the colony were observed around the strains indicating the enzymatic degradation of chitin (Rodriguez-Kabana *et al.*, 1983).

Cellulase production

Cellulase activity was tested on M9 medium (Miller, 1972) supplemented with yeast extract (0.12% w/v) and carboxyl-methylcellulose (CMC) (1% w/v) (Cattelan *et al.*, 1999). Strains surrounded by clear halo after 8 days of incubation at

28°C were considered as positive for cellulase production.

Pectinase production

Pectinase production was assayed by the protocol described by Cattelan *et al.*, (1999). Strains were inoculated on M9 medium (Miller, 1972) amended with pectin (4.8 g/L) were incubated at 28°C for two days and subsequently flooded by 2M HCl. The clear halo around the colonies indicated pectinase production.

Protease production

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. Digest of casein 5.0, Yeast extract 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Strains were streaked and incubated at 30°C and zone of clearance around the colony indicating the enzymatic degradation of protease (Chaiharn, 2008).

Catalase production

Catalase test was performed by adding three to four drops of H₂O₂ on actinomycetes strains which were grown for 48h on trypticase soya agar medium (Trypticase 15, Phytone 5.0, NaCl 5.0, Agar 15.0, Distilled water 1000ml). The effervescence indicated catalase activity (Schaad, 1992).

Lipase production

Actinomycetes strains were grown on starch casein agar medium amended with egg yolk. After 24h of incubation clear zones around the colony indicated positive for lipase activity (Smibert and Krieg, 1981).

Gelatinase production

Inoculum of 18-24h old test actinomycetes was inoculated into tubes containing nutrient gelatin. The inoculated tubes along with uninoculated control were incubated at 25°C for 7-10 days. After incubation, the tubes were placed in refrigerator for 15 to 30 minutes. Afterwards, hydrolyzed gelatin showed liquified medium against the uninoculated control medium in solid form (Stapp, 1953 and Shejul, 1999).

Results and Discussion

All strains are Gram-positive, slow growing mycelial forms, producing diverse pigments on starch casein agar medium with aerial and expansive growth (Table 1). All 54 strains were designated as OUA1 to OUA54 and screened against phytopathogens *C. capsici* and *F. oxysporum*. Only 25 strains showed antifungal activity against *C. capsici*. Among these 25 strains only 13 strains (OUA3, OUA5, OUA7, OUA8, OUA9, OUA12, OUA14, OUA17, OUA18, OUA27, OUA40, OUA41 and OUA50) were antagonistic to *F. oxysporum* (Table 1). Strains OUA5, OUA17 and OUA18 showed maximum inhibition of 85.7% against *C. capsici*. OUA14 and OUA50 strains exhibited maximum of 75% growth inhibition against *F. oxysporum* (Table 1 and Figure 1). The percentage inhibition of 25 strains ranged from 20% to 85.7% against *C. capsici*. Inhibition against *F. oxysporum* ranges from 25-75%. The inhibition was lowest (20%) against *C. capsici* by a OUA28 and OUA29 while OUA18 was less inhibitory (20%) to *F. oxysporum*.

Grey colored powdery colony of the strain OUA5 produced yellow colour pigment, while the expansively growing OUA17 colony produced pink color pigments, no pigment was seen in OUA18. Isolates OUA28 produced yellow pigment and blue colored OUA29 produced blue color pigmentation (Table 1).

These potent strains of antifungal actinomycetes have the ability to produce enzymes such as amylase and protease (Table2). Strains OUA31 and OUA38 possessed only amylase and protease activities. The OUA50 showed amylase, protease and catalase activities. Among the 25 strains, OUA36 failed to show any of the enzyme activities while OUA17 produced only amylase but not protease. Strains OUA3, OUA5, OUA18, OUA40 possessed all the enzyme activities viz., amylase, chitinase, protease, catalase, lipase, in addition to gelatin hydrolysis. Strain OUA3 and OUA18 failed to hydrolyse gelatin, OUA8 and OUA12 showed no catalase activity.

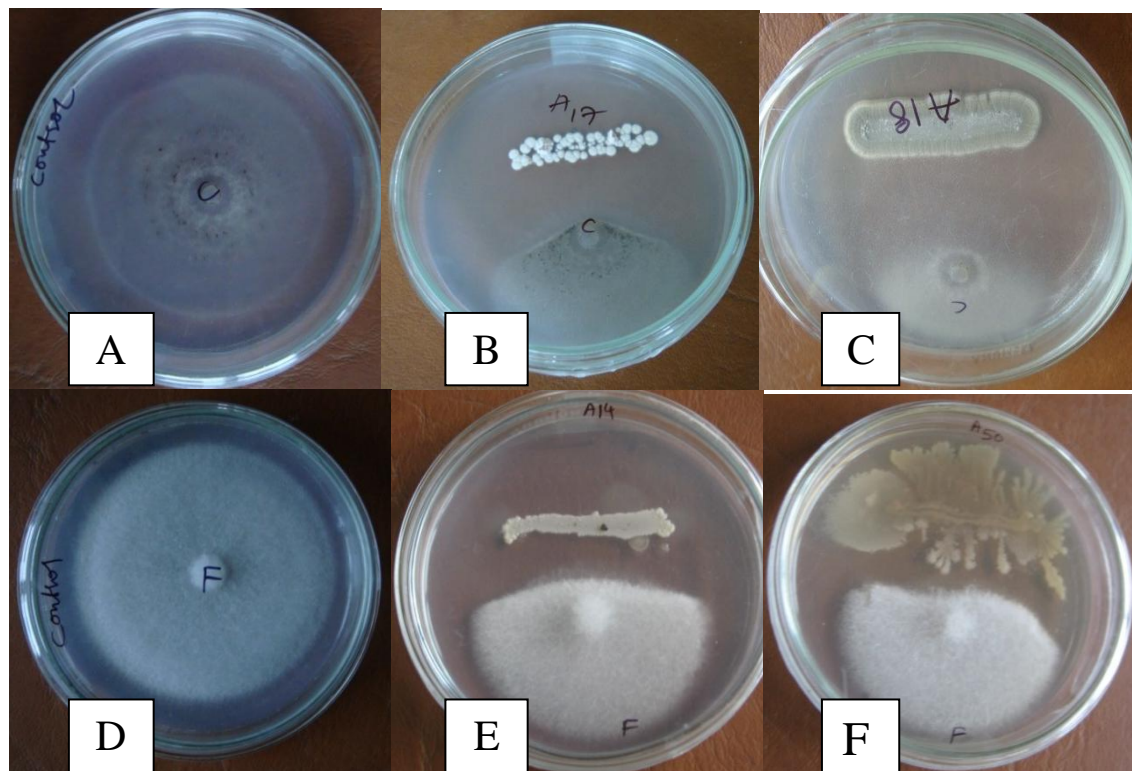
Gram positive bacteria gaining importance, since they are able to synthesize chemically diverse compounds with wide range of biological activities. Actinomycetes are unparalleled source of bioactive metabolites such as antibiotics, plant growth factors and enzymes (Shahidi *et al.*, 2004, Narendrakumar *et al.*, 2010). Among the 54, 25 actinomycetes strains isolated from rhizosphere soils of chilli plants were antifungal against the plant pathogens, *C. capsici* and *F. oxysporum*. Exploring soils for actinomycetes having antifungal activity against fungal phytopathogens has been a continuous effort since a long time with remarkable success.

Table.1 Actinomycetes strain number, Color of strain, Growth, Pigment production, Inhibition percentage against *Colletotrichum capsici* and *Fusarium oxysporum*

S. No.	Color	Growth	Pigmentation	Growth inhibition (%)	
				<i>C. c.</i>	<i>F. o.</i>
OUA3	Grey	Exp	Yellow	80.0	57.5
OUA5	Grey	Pow	Yellow	85.7	47.5
OUA7	LG	Pow	Black	48.5	55.0
OUA8	DC	Pow	No pigment	77.1	62.5
OUA9	White	Exp	Yellow	42.8	37.5
OUA12	Grey	Pow	No pigment	65.7	52.5
OUA14	Cream	Exp	No pigment	65.7	75.0
OUA15	Grey	Pow	Brown	54.2	-
OUA16	White	Exp	No pigment	48.5	-
OUA17	LP	Exp	Pink	85.7	67.5
OUA18	Grey	Pow	No pigment	85.7	25.0
OUA27	LG	Exp	Green	77.1	55.0
OUA28	DW	Pow	Yellow	20.0	-
OUA29	Blue	Exp	Blue	20.0	-
OUA30	DC	Pow	Yellow	48.5	-
OUA31	White	Exp	Brown	65.7	-
OUA32	Grey	Exp	No pigment	57.1	-
OUA33	White	Pow	Brown	71.4	-
OUA36	White	Exp	No pigment	28.5	-
OUA37	Cream	Exp	Yellow	77.1	-
OUA38	DW	Pow	Brown	62.8	-
OUA39	White	Pow	Brown	30.0	-
OUA40	LP	Exp	Pink	77.1	65.0
OUA41	Grey	Exp	Black	40.0	57.5
OUA50	Pink	Exp	Pink	80.0	75.0

St. No.- Actinomycetes strain number, **C. c.-** *Colletotrichum capsici*, **F. o.-** *Fusarium oxysporum*, **LG-** Light grey, **DC-** Dark cream, **LP-** Light pink, **LG-** Light green, **DW-** Dull white, **Exp-** Expansive, **Pow-** Powdery, - - No inhibition.

Figure.1 Antagonistic activity of Actinomycetes strains



A) *C. capsici* (control), **B)** OUA17 and **C)** OUA18 against *C. capsici*, **D)** *F. oxysporum* (control), **E)** OUA14 and **F)** OUA50 against *F. oxysporum*.

Table.2 Enzyme activities of actinomycetes strains

St. No	Amy	Chit	Prot	Cat	Lip	G H
OUA3	+	+	+	+	+	-
OUA5	+	+	+	+	+	+
OUA7	+	+	+	-	+	+
OUA8	+	+	+	-	+	+
OUA9	+	+	-	+	-	-
OUA12	+	+	+	-	+	+
OUA14	+	+	+	+	+	+
OUA15	+	-	+	+	-	+
OUA16	+	+	+	+	-	-
OUA17	+	-	-	-	-	-
OUA18	+	+	+	+	+	-
OUA27	+	+	+	-	-	-

OUA28	+	+	+	-	-	+
OUA29	+	+	+	-	-	-
OUA30	+	+	+	-	-	+
OUA31	+	-	+	-	-	-
OUA32	+	+	+	-	+	-
OUA33	+	+	+	+	+	-
OUA36	-	-	-	-	-	-
OUA37	+	+	+	-	-	-
OUA38	+	-	+	-	-	-
OUA39	+	+	+	-	-	+
OUA40	+	+	+	+	+	+
OUA41	+	+	+	-	+	+
OUA50	+	-	+	+	-	-

St. No. - strain number, **Amy**- Amylase, **Chit**- Chitinase, **Prot**- Protease, **Cat**- Catalase, **Lip**- Lipase, **G H**- Gelatin hydrolysis, ‘+’ - Positive, ‘-’ - Negative

Aghighi *et al.*, (2004) obtained 14 active isolates out of 110; 10 isolates by Bonjar *et al.*, (2005) and 98 antifungal isolates out of 316 actinomycetes from different localities of Garhwal region, Uttarakhand, (Bharti *et al.*, 2010). Bailo *et al.*, (2003) reported 335 soil actinomycetes with only 230 antimicrobial isolates under different pH and salinity conditions. Soil actinobacteria exhibited varying percentage of antifungal activity depending on their habitat, nature of soil etc. The rhizosphere of chillies supported 54 different actinobacteria with about 50% antifungal strains against the plant fungal pathogens. It has long been shown that antagonistic actinomycetes are effective protectants against soil-borne fungal pathogens (Crawford *et al.*, 1993).

Among the 25 antifungal actinomycetes, 96% (24) were amylytic, 88% were proteolytic, 76% (19) were chitinolytic results that the actinomycetes isolated

and 44% (11) exhibited lipolytic activity. The isolates OUA5 and OUA40 possessed all the enzymes while OUA36 showed no enzymes activity. It is clear from the present results that approximately 96% of the potent antifungal strains produced one or more enzymes conforming earlier reports (Tsujibo *et al.*, 2003, Muiru *et al.*, 2007, Mohan and Singara Charya, 2012). The present results indicate that actinomycetes possess the potential to produce broad range of enzymes which may be due to the result of natural selection of microorganisms in order to survive in the competing environment (Arjit *et al.*, 2012). No strain has produced the enzymes cellulase and pectinase. Patke and Dey (1996) who reported protease activity in isolates of actinomycetes, Shejul (1999) reported gelatinase activity in isolates of actinomycetes. Amylase activity was reported by Abraham and Herr (1964). It appears from the present from the rhizosphere soil many serve as an

important resource for screening useful enzymes and bioactive metabolites.

Antifungal nature against pathogenic fungi has been attributed to multiple phenomena including antibiosis and parasitism. Production of extracellular enzymes such as chitinases, proteases and other hydrolytic enzymes may act against the fungal cell wall that consists of chitin and other polysaccharides. Since chitinolytic activity is a common feature of actinomycetes from tropical soils (Gupta *et al.*, 1995; Gomes *et al.*, 1999), majority of strains (19) in this study are capable of producing chitinases besides other hydrolytic enzymes suggesting their utility in biological control of phytopathogenic fungi in contrast to the fresh water actinomycetes which lack the enzyme chitinase (Mohan and Singara Charya, 2012).

Cellulolytic activity of actinomycetes is well known (Amira *et al.*, 1989) and has received considerable attention in recent times (Arunachalam *et al.*, 2010). However, all the tested isolates in the present study failed to produce the enzyme cellulase. The actinomycetes inhabiting the rhizosphere may be more adopted to prevent the deleterious microorganisms like pathogenic fungi rather than degrading the organic matter in soil. Therefore these isolates produced chitinases and other hydrolytic enzymes instead of cellulases and the rhizosphere may serve as a repository of potential biocontrol agents.

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