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Biochemical characterization of Actinomycetes isolated from rhizosphere soils of groundnut in Andhra Pradeh

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ABSTRACT

Roving survey was conducted and collected Actinomycetes isolates in major groundnut growing areas of Andhra Pradesh (10 districts) *viz.*, Anantapuramu, YSR Kadapa, Kurnool, Chittoor, SPSR Nellore, Prakasam, Guntur, West Godavari, Vizianagaram and Srikakulam during Kharif, 2016. During the survey, 180 rhizospheric soil samples were collected and isolated a total number of fifty morphologically different Actinomycetes isolates. Basic identification of Actinomycetes was done by visual observation, general morphology, spore formation, colony morphology, an earthy odouri and including Gram stain. The isolates were characterized based on different biochemical tests. The enzymativ activity of isolates was studied to know the efficacy against stem rot pathogen *Sclerotium rolfsii* in groundnut.

Key words : *Actinomycetes, Biochemical, Characterization, Groundnut*

Introduction

Globally, groundnut is cultivated throughout the tropical, sub tropical and warm temperate region of the world (Nwokolo, 1996). Ground nut stem rot caused by *Sclerotium rolfsii* Sacc. damages the crop with a pod loss of 30-40 per cent especially near harvest time (Johnson and Subramanyam, 2000) . Stem rot pathogen attacks the germinated groundnut seedlings and causes wilt and all the plant parts are susceptible to *S. rolfsii* but stem infection is the most common and destructive one (Deepthi, 2013). Once a field is infested, the pathogen may survive in the soil for many years. The degree of loss caused by the pathogen varies depending on the host cultivar, the pathogen subspecies and environmental conditions. Because of this reason, the management of stem rot disease becomes very difficult. The soil borne nature of the pathogen and lack of disease resistance in existing commercial cultivars make the situation further worse. Majority of the existing biocontrol agents for management of soil-borne diseases, were isolated from the rhizosphere. Biological control with potential Actinomycetes is receiving greater attention all over the world. Among Actinomycetes, *Streptomyces* being root-colonizing and rich producer of secondary metabolites become one of the important promising group of antagonists. Studies were conducted on the collection and isolation of Actinomycetes from different groundnut growing areas of Andhra Pradesh to identify potential isolates against stem rot pathogen *Sclerotium rolfsii.* Soil actinomycetes have revealed their wide antifungal activity (Tinatin and Nuzrat 2006). Actinobacteria

are one of the important promising group of antagonists and root-colonizing microbes which survives in soils. Among them, the genera *Streptomyces*, a high guanine-plus-cytosine (G+C) content (69 – 78%) soil-dwelling Gram-positive bacterium, undergoes a complex cycle of morphological differentiation leading to sporulation, production of diverged bioactive compounds including useful antibiotics, pigments, siderophores, chitinases and phytohormones with phosphate solubilizing abilities (Stackebrandt *et al*., 1997).

The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes like chitinases and â-1, 3-glucanase which involves in the lysis of fungal cell walls. Such a plethora of enzymes, antibiotics and secondary metabolites make them formidable competitors in natural environments as well as very attractive organisms for biotechnological purposes (El-Katatny *et al*., 2001).

Methodology

Collection of Soil sample Soil samples were collected at the rhizosphere of healthy groundnut plants adjacent to stem rot affected plants. After soil sample taken, it was packed in polyethylene bags to minimize moisture losses during transportation. Samples were air dried for one week then were crushed and sieved. The sieved soil samples were pretreated by mixing 1g of soil with 0.1g Calcium carbonate and incubated at 37 °C for 2-5 days. This pretreatment enhances the population of *Streptomycetes spp*. in soil samples (Boroujeni *et al.,* 2012).

Isolation and identification of Actinomycetes by soil dilution plate method

Ken Knight's Agar medium was used for the isolation of Actinomycetes (Allen, 1953). 10 g soil sample was suspended in 100 ml sterile water (10%) and agitated for 30 min at 420 rpm. These suspensions were considered as 10^{-1} dilution. From 10^{-1} suspension, took the 1 ml supernatant transferred to 9 ml of sterile distilled water and subsequently serially diluted to 10^{-3} , upto 10^{-6} etc. From the required dilution, 0.1 ml suspension was drawn and plated over the surface of Ken knight's medium by spread plate technique (Allen, 1953). All the plates were incubated at 28°C ± 2°C for 5 days. Colonies of Actinomycetes on agar plates were picked up on the basis of their morphological characteristics and isolated as pure culture by routine microbiological methods and maintained on Ken knight agar slants and as 20% (w/v) glycerol stock. The slant cultures were stored at room temperature and kept in dark while glycerol stocks were kept in freezer at -20 ºC and -80 ºC.

Results and Discussion

Isolation of different soil Actinomycetes

Roving survey was conducted to collect the isolates of Actinomycetes in major groundnut growing areas of Andhra Pradesh in 10 districts *viz.*, Anantapuramu, YSR Kadapa, Kurnool, Chittoor, PSR Nellore, Prakasam, Guntur, West Godavari, Vizianagaram and Srikakulam during the kharif, 2016. During the survey 180 rhizospheric soil samples were collected from healthy roots of a plant present nearby disease affected plant and isolated a total number of fifty morphologically different Actinomycetes by soil dilution plate technique using Ken Knight's Agar medium (Table 1). Basic identification of Actinomycetes was done by visual observation, general morphology, spore formation, colony morphology including Gram stain and biochemical tests and an earthy odour. Among other

Table 1. Actinomycetes isolates collected from different districts of Andhra Pradesh

S. No.	District	Mandal	Isolate
1	Anantapuramu	Kalyandurgam	Kyd
2		Kadiri	Kdr
3		Narpala	Npl
4	Y S R Kadapa	Lakkireddipalli	Lrp
5		Vempalli	Vpl
6		Tallapalle	Tpl
7	Kurnool	Pattikonda	Pkd
8		Yammiganur	Ymn
9		Gonegandla	Ggd
10	Chittoor	Molakalacheruvu	Mkc
11		Chandragiri	Cdg
12		Narayanavanam	Nyv
13	S P S R Nellore	Kavali	Kvl
14		Sullurpet	Slp
15	Prakasam	Kothapatnam	Kpt
16	Guntur	Cherukupalli	Crp
17	West Godavari	Chintalapudi	Clp
18	Vizianagaram	Merakamudidam	Mmd
19	Srikakulam	Tekkali	Tkl
20		Ranastalam	Rsl

inhabitants of soil samples the isolates identified by zones of growth inhibition as the major evidence of antibiotics production.Among the fifty, twenty isolates were selected based on the growth rate of isolate on culture medium and zone of growth inhibition. All the twenty isolates further identified as Actinomycetes by general biochemical and morphological characters and proved that all were acid-fast negative, Gram stain positive and aerobic with aerial and substrate mycelia of different colors with spiral spore chains.

Gram staining

A smear of the Actinomycetes isolate was prepared on a clean glass slide and the smear was allowed to air-dry and then heat-fixed. The heat-fixed smear was flooded with crystal violet(AppendixI) and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 s. After washing with water, the smear was dried with tissue paper and examined under oil immersion (100 x) (Williams *et al*., 1989). The isolated Actinomycetes were identified by biochemical properties performing various biochemical tests according to the Bergey's manual. All tests were performed at room temperature.

Enzymatic screening of isolates

Chitinases production test

Colloidal chitin agar media (Appendix I) plates were streaked with the Actinomycetes isolates and incubated for 2 weeks at 28°C and observed for the presence of halo zone around the vicinity of colony of Actinomycetes (Hsu and Lockhood, 1975).

Cellulases production test

Minimal medium agar with CMC in the media plates were streaked with the Actinomycetes isolates and incubated for 2 weeks at 28°C and observed for the presence of clear zone around the colony growth of Actinomycetes.

Lipases production test

Tween 80 agar plates were streaked with the Actinomycete isolates and incubated for 5 days at 28 °C and observed for the presence of halo of white precipitate opaque zone around the colony of Actinomycetes.

Proteases production test

The Actinomycete isolates were streaked on Casein agar plates and incubated at room temperature (28±2 °C) for 5 days. The plates were observed for the clear zone of casein hydrolysis around the growth of Actinomycetes.

Starch hydrolysis

Actinomycetes isolates were streaked on solidified starch agar medium and incubated for 3 days at 28°C. At the end of incubation, the plates were flooded with Lugols solution (Appendix I) for 30 seconds and then drained. The plates were examined for the hydrolysis of starch by the production of clear zone around the growth of Actinomycetes or decolorization against the blue colour background.

HCN production test

Actinomycetes isolates were grown on Ken knight's agar. One sterilized sheet of filter paper was soaked in reagent solution (0.5% picric acid+ 2% sodium carbonate) for 1 min. and attached to the undersurface of the Petridish lid. The lids were replaced and sealed with parafilm and incubated for 4 days at 28 ºC. Development of cream, light brown or reddish brown color on the filter paper indicated for

Table 2. Bio chemical characterization of potential Actinomycetes

S. No.	Isolate	Cellulase assay	Lipase assay	Protease assay	Chitinase assay	Amylase assay	HCN production assay
1	Ggd	+	+	+	+	+	+
2	Kyd	+	+	+	+	+	-
3	Kdr	+	+	+	+	+	-
4	Lrp	+	-	-	+	+	-
5	Mkc	+	-	-	+	+	-

+ Positive reaction; - Negative reaction

positive for HCN production.

To understand the biochemical activity of all the strains various enzymatic screening studies were carriedout. These are the cellulase, lipase, protease, chtinase, HCN production and amylase assays.

Each potential isolate was inoculated onto a specific medium for enzymatic assays and the results indicated in Table 2. In cellulase assay all the five isolates showed positive reaction. The isolates Ggd, Kdr, Kyd, Lrp and Mkc formed a clear zone of 10 mm. The isolates Ggd, Kdr and Kyd showed positive reaction in lipases assay. While negative reaction was showed by Lrp and Mkc. In proteases assay the isolates Ggd, Kdr and Kyd showed positive reaction. Negative reaction was showed by Lrp and Mkc. In chitinase assay all the five isolates showed positive reaction. The isolate Ggd formed a clear zone of 20 mm while Kdr and Kyd with 15 mm, Lrp and Mkc with less than 10 mm. All the five isolates screened for HCN production assay. Observed light brown discoloration on filter paper for the isolate GGD while remaining four isolates Kdr, Kyd, Lrp and Mkc formed a cream colour discolouration. It was observed that all the isolates produced a clear zone for amylases test while the diameter of clear zone was around 20 mm for Ggd, Kdr and Kyd whereas less than 20 mm for Lrp and Mkc. In the present study the isolate Ggd was positive for all the assays showing its highest enzymatic activity. The isolates Kdr and Kyd were also good in enzymatic activity showed positive for all the assays except for HCN production assay. The isolates Lrp and Mkc were positive for only cellulase, chitinase and amylase assays.

All these results have shown same pattern of results obtained by previous works reported by several researchers. The enzymatic activity of *Streptomyces* strains plays an important role in the biocontrol of stem rot disease and the plant growth promotion is a good outcome.

The isolates of *Streptomyces* have the ability to produce a diverse production of antimicrobial secondary metabolites. This may additionally allow them to compete for space and nutrients that are exuded by plants. In addition to contributing to plant protection, members of this genus are frequently found to contribute to plant growth promotion under both ambient and stressful environmental conditions, such as high salinity (Chaurasia *et al*., 2018 ; Patel *et al*., 2018). These additional benefits could form the basis for highly desirable biocontrol agents that can both enhance plant growth and protect the plant against disease.

EL-Tarabily *et al*. (2006) reported that *Streptomyces viridodiasticus* antagonistic to *Sclerotinia minor* which caused extensive hyphal plasmolysis and cell wall lysis and significantly reduced the growth of the pathogen *in vitro* with the production of high levels of chitinase and β -1,3-glucanase along with antifungal metabolites. In addition, significant reduction of lettuce basal drop disease incidence observed with competent S. *viridodiasticus* under controlled glasshouse conditions. The production of these enzymes was therefore used as the criteria for selection of potential biocontrol agents against *S.rolfsii*. Hydrolysis of starch was evaluated by using the media of Gordon *et al*. (1974).

Streptomyces could be used in place of conventional chemical treatments as potential biocontrol agents as these are abundant in soil and have been shown to suppress a range of phytopathogenic organisms both *in vitro* and *in vivo* (Viaene *et al*., 2016).

Chen *et al*., (2016) reported that the ability of *Streptomyces* to penetrate plant roots eventually led to an endophytic lifestyle. Fluorescent microscopy has shown that these can exist endophytically within the roots of several different plant species, including lettuce, wheat and pea.

Jacob *et al*. (2018) Stated that importantly for a biological control agent to be effective, it must showcase multiple mechanisms of pathogen population control which was evident from the production of HCN, siderophores and also the role of the antifungal metabolites.

These secondary metabolites are known to have a diverse range of activities and have been used for a wide range of applications including antibacterial and antifungals compounds (Olanrewaju and Babalola, 2019).

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