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Isolation of plant growth promoting actinobacteria from the rhizosphere of finger millet and cowpea

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Abstract
Actinobacteria commonly inhabit the rhizosphere, being an essential part of this environment due to their interactions with plants. Such interactions have made possible to characterize them as plant growth-promoting rhizobacteria (PGPR). As PGPR, they possess direct or indirect mechanisms that favor plant growth. Actinobacteria improve the availability of nutrients and minerals, synthesized plant growth regulators. A total of forty actinobacterial isolates were obtained from rhizosphere of finger millet and cowpea. The isolates were characterized morphologically and biochemically. Biochemical characterization of forty isolates of actinobacterial isolates, twelve isolates were positive for casein hydrolysis, eleven isolates were positive for tyrosine, twenty isolates were positive for H_2S production, seven isolates were positive for gelatin liquefaction, twenty three isolates were positive for indole test, three isolates were positive for methyl red test, five isolates were positive for Voges Proskauer test, ten isolates were positive for urease production, eight isolates were positive melanin production and seven isolates were positive for citrate production, and negative for HCN production. The average count of actinobacteria enumerated on five different media (actinobacteria isolation agar, humic acid vitamin agar, starch casein agar, Kuster's agar and ISP-4 media) were 7.75 x 10^3, 6.01 x 10^3, 15.86 x 10^3, 3.04 x 10^3 and 7.55 x 10^3 CFU g^{-1} of sample respectively. The highest population of 29.00 x 10^3 CFU g^{-1} was observed in the rhizosphere of cowpea compared to all the other samples used for the isolation. Among the five media used for the enumeration of actinobacteria starch casein agar was found best for the growth of actinobacteria.

Keywords: Actinobacteria, biochemical characterization, starch casein agar

Introduction
Actinobacteria commonly inhabit the rhizosphere, being an essential part of this environment due to their interactions with plants. Such interactions have made possible to characterize them as plant growth-promoting rhizobacteria (PGPR). As PGPR, they possess direct or indirect mechanisms that favor plant growth. Actinobacteria improve the availability of nutrients and minerals, synthesized plant growth regulators, and specially, they are capable of inhibiting phytopathogens. Different activities that are performed by actinobacteria have been studied, such as phosphate solubilization, siderophores production, and nitrogen fixation. Furthermore, actinobacteria do not contaminate the environment; instead, they help to maintain the biotic equilibrium of soil by cooperating with nutrients cycling. Actinobacteria are the most widely distributed group of microorganisms and occur as saprophytes in soil (Takizawa *et al.,* 1993) [11]. They are Gram-positive, prokaryotic microorganisms; their growth and proliferation in soil is influenced by number of factors such as soil type, organic matter content, salinity, relative moisture content, (Ilic *et al.,* 2007) [5]. *Streptomyces* and other Actinobacteria are major contributors to biological buffering of soils and play an important role in organic matter decomposition (Dhingra and Sinclair, 1995). They are able to produce spores, which help in dissemination and resistance to many adverse conditions (Goodfellow and Williams, 1983; Chater, 1993) [3, 2].

For isolation of soil actinobacteria, selection of media is important for understanding their ecological properties and for discovery of novel strains which can produce useful bioactive secondary metabolites. Numerous media have been described for the isolation of actinobacteria from soil and other natural materials. Use of isolation media with high carbon to nitrogen ratio and resistant complex carbon and nitrogen sources, *e.g.,* starch, casein, chitin, humic acid *etc.,* is suitable for isolation and enumeration of actinobacteria (Gray and Williams, 1971) [4].

The increasing emergence of new diseases and pathogens, and the antibiotic resistance in recent years have caused an interest in searching for new biologically active compounds, thus the studies on distribution and diversity of actinobacteria are crucial in discovering new strains and to explore ecological niches in different regions worldwide. The aim of this study was to isolate actinobacteria from the cowpea and finger millet rhizosphere, characterize these isolates on the basis of morphological, biochemical characterise and compare different media for enumeration of actinobacteria

Material and Methods

Collection of soil samples for the isolation of actinobacteria

The soil samples were collected from the rhizosphere of cowpea and finger millet crop fields, at University of Agricultural Sciences, Bangalore, in labelled sterile polythene bags and brought to the laboratory. and preserved in a refrigerator condition (@ 4 °C) for further studies. Along with rhizosphere soil the different organic manure samples like vermicompost, goat droppings, forest litter soil and farm yard manure (FYM) were also used for isolation of actinobacterial isolates.

Isolation and comparison of media for the enumeration of actinobacteria

Isolation of actinobacteria was done by standard dilution plate technique after serial dilution of soil samples. Five different media were used for isolation and enumeration of Actinobacteria *viz.,* actinomycetes isolation agar, starch casein agar, International *Streptomyces* Project -4 (ISP-4), humic acid vitamin agar and Kuster's agar. Approximately 20 ml of respective medium were poured into the sterilized petri plates and kept for incubation for ten days. Further, the actinobacterial isolates were purified by repeated streaking on fresh media and preserved on starch casein agar slants for further studies.

Biochemical characterization

The biochemical characterization was essentially carried out for all the isolates as per the standard procedures outlined by Cappuccino and Sherman (2005) [1]. The tests conducted are described below.

Casein hydrolysis

The actinobacterial isolates were streaked on skim milk agar plates and incubated at room temperature (25-37 ºC). The hydrolysis of casein was indicated by the presence of clear zones surrounding the colonies.

Melanin production

Melanin production was determined by using peptone yeast extract iron agar (ISP medium 6) amended with 0.1per cent yeast extract as per the procedure given by Shirling and Gottlieb (1966) [10]. Cultures forming diffusible pigments of greenish brown to brown or distinct brown pigment after two days were recorded as positive for melanin production.

Tyrosine utilization

Tyrosine utilization was determined by using tyrosine agar medium (ISP 7) as per the procedure of Shirling and Gottlieb (1966) [10]. Formation of clear zones surrounding the colonies indicates the utilization of tyrosine.

N free agar medium

Actinobacterial isolates were streaked on N free Jensen's medium (Norris and Chapman, 1968) [9] to test for their ability to grow in the absence of fixed nitrogen compounds. Growth indicated the ability of the isolates to fix atmospheric nitrogen.

Hydrogen sulphide production

SIM (Sulfide-Indole-Motility medium) was prepared and all the test cultures were stabbed in to the medium. SIM medium contains peptone and sodium thiosulfate as the sulfur substrate, ferrous sulfate ($FeSO_4$) as H_2S indicator. Ferrous ammonium sulfate in the medium serves as an indicator by combining with the hydrogen sulfide gas forming an insoluble black ferrous suifide precipitate that is seen along the line of the stab inoculation which is a positive indication of H_2S production.

Gelatin liquefaction

Nutrient gelatin tubes were prepared and test cultures were inoculated and incubated for 48 hours. After incubation all the gelatin slants were placed in a refrigerator at 4℃ for 30 minutes. All the inoculated tubes were examined to see whether the medium has solidified or remained liquid. If they remained liquid it was taken as positive.

HCN production

HCN was estimated as per the method described by Lorck (1948) [8]. The actinobacterial isolates were grown in Bennett agar amended with glycine (4.4 g). A sheet of Whatman filter paper no. 1 (8 cm dia.) was soaked in 1 per cent picric acid in 10 per cent sodium carbonate solution (filter paper and picric acid were sterilized separately) for a minute and placed in the Petri dish.

The plates were sealed with paraffin film and incubated at 28 ± 2 ºC for four days. Development of reddish-brown color on the filter paper indicated positive for HCN production.

Urease test

The actinobacterial isolates were inoculated to the test tube containing 5 ml of sterilized urea broth with phenol red as pH indicator and incubated at 30± 2°C for 24 - 48 hours. The development of dark pink color was recorded as positive for urease activity.

Citrate utilization test

The actinobacterial isolates were streaked on the slants containing Simmon's citrate agar with bromothymol blue as an indicator and were incubated at 37± 2°C for 48 hours. After incubation, slants were observed for the growth and formation of blue color, and it was considered as positive for the citrate utilization test.

Indole production

The actinobacterial isolates were inoculated to the tubes containing sterilized tryptone broth and were incubated at 30 ± 2°C for 48 hours. After the incubation period, 10 drops of Kovac's reagent was added to each tube. The development of red color was taken as positive for the indole production.

Methyl red test

The actinobacterial isolates were inoculated to the pre-sterilized tubes containing MR-VP broth and incubated for 48

hours at 37± 2°C. After incubation, 5 drops of methyl red indicator were added to each tube and gently shaken. The formation of red color was taken as positive and formation of yellow color was taken as negative for the test.

Voger - Proskauer test

The actinobacterial isolates were inoculated to the pre-sterilized tubes containing MR-VP broth, and incubated at 37°± 2°C for 48 hours. After incubation, 10 drops of Barritt's reagent A was added and gently shaken followed by addition of 10 drops of Baritt's reagent B. The development of rose color in the broth was considered as positive for the test.

Results and Discussion

The soil samples were collected from the rhizosphere of cowpea and finger millet crop fields, at University of Agricultural Sciences, Bangalore, along with soil the different organic manure samples like vermicompost, goat faeces, forest litter soil and FYM were also used for isolation of actinobacterial isolates (Table 1). Isolation of actinobacteria was carried using different nutrient media. The average counts of actinobacteria enumerated on five different media *viz*., actinobacteria isolation agar, humic acid vitamin agar, starch casein agar, Kuster's agar and ISP-4 media were 7.75 x 10^3, 6.01 x 10^3, 15.86 x 10^3, 3.04 x 10^3 and 7.55 x 10^3 CFU g^{-1} of the samples respectively. Significantly the highest population was observed in the rhizosphere of cow pea on starch casein agar media compared to all the other samples and media. Lowest population (5.20 x 10^3 CFU g^{-1}) was recorded in the forest litter soil sample (Table 2). Among the media used, the population was more on starch casein agar followed by ISP-4 media (International *Streptomyces* Project - 4 media). Among the different media used, starch casein agar was found best for the growth of actinobacteria. Jiang *et al.* (2016) [6] have described different types of selective media used for isolation of actinobacteria from different habitats *viz.* YIM 14 improved Czapek medium, YIM 17 glycerol asparagine medium and YIM 21 oatmeal medium for isolation of thermophilic actinobacteria, YIM 6 Starch-casein medium, YIM 17 glycerol asparagine medium, YIM 47 soil extracts medium, T3 medium and Horikoshi medium for isolation of halophilic, alkalophilic and acidophilic actinobacteria, Water yeast extract medium and Sodium propionate medium for isolation of endophytic actinobacteria and YIM 7 HV medium, YIM 7 HV medium and YIM 212 histidine-raffinose medium for isolation of rare actinobacteria. Biochemical characterization was carried out for all the 40 actinobacterial isolates obtained from different samples. Biochemical characterization of actinobacterial isolates showed that out of forty isolates, 12 isolates were positive for casein hydrolysis, 11 isolates for tyrosine, 20 isolates for H_2S production, 7 isolates for gelatin liquefaction, 23 isolates for indole test, 3 isolates for methyl red test, 5 isolates for Voges Proskauer test, 10 isolates for urease production, 8 and 7 isolates could produce melanin and citrate respectively, and negative for HCN production. Similar results were observed by Kekuda *et al.* (2012) [7] w.r.t. staining and biochemical characteristics of the actinomycete isolate SRDP-H03 isolated from the rhizosphere soils of Hosudi, Shimoga (Karnataka). The isolate was Gram positive and non-acid fast and the spore arrangement was flexous type. The isolate showed positive result for amylase, cellulase, catalase, gelatinase and citrate production. Production of oxidase, caseinase and H_2S were not detected. The isolate fermented glucose and fructose with acid production and galactose and lactose with alkali production. Acid or alkali production was not observed during maltose fermentation. Based on the cultural and microscopic characteristics, the isolate SRDP-H03 was assigned to the genus *Streptomyces.*

Table 1: Particulars of the samples used for isolation of actinobacteria

Sl. No.	Sample	Place	Crop	Geographic position of the sample site	
				Latitude (N)	Longitude (E)
1.	Red soil	Dry land, UAS(B), GKVK, Bengaluru	Finger millet	13.083922	74.57455
			Cowpea	13.087077	77.56490
2.	Red soil	Krishimela site, UAS(B), GKVK, Bengaluru	Finger millet	13.083924	74.57455
			Cowpea	13,085135	77.57094
3	Red soil	AICRP, UAS(B), GKVK, Bengaluru	Cowpea	13.075363	77.56194
				13.076048	77.56292
				13.076043	77.56091
4	Farm yard manure	ZARS, UAS(B), GKVK, Bengaluru	-	13.075462	77.56132
5	Vermicompost	ZARS, UAS(B), GKVK, Bengaluru	-	13.076034	77.56222
6.	Goat droppings	ZARS, UAS(B). GKVK, Bengaluru	-	13.075362	77.56053
7.	Forest soil	BRT hills Chamarajanagar	-	11.580149	77.1000

Note: UAS(B) - University of Agricultural Sciences, ZARS – Zonal Agricultural Research Centre, AICRP- All India Coordinate Research Project, GKVK- Gandhi Krishi Vigyana Kendra

Table 2: Population of actinobacteria (No. X 10^3 CFU g^{-1} soil) on different growth media

SI. No.	Sample	Crop	Media				
			AIA	HVA	SCA	KA	ISP-4
1	Red soil	Ragi	9.50^{ab}	8.00^{a}	14.70^{bc}	3.06^{e}	8.60^{b}
		Cowpea	8.50^{c}	7.20^{bc}	9.00^{d}	3.03^{e}	6.70^{c}
2	Red soil	Ragi	9.00^{bc}	7.50^{ab}	27.70^{a}	4.03^{c}	11.80^{a}
		Cowpea	9.80^{a}	7.30^{b}	28.00^{a}	4.23^{b}	12.10^{a}
3	Red soil	Cowpea	9.60^{ab}	7.70^{ab}	29.00^{a}	5.03^{a}	8.50^{b}
4	Farm yard manure	-	7.80^{d}	6.70^{c}	14.20^{c}	3.33^{d}	5.70^{d}
5	Vermicompost	-	6.50^{e}	3.60^{d}	8.00^{de}	2.83^{g}	5.60^{d}
6	Goat faeces	-	5.30^{f}	3.10^{de}	7.00^{e}	2.33^{h}	5.30^{d}
7	Forest soil	-	3.80^{g}	3.00^{e}	5.20^{f}	2.93^{f}	3.70^{e}
	Average counts (Population)	-	7.75	6.01	15.86	3.40	7.55

Note: Mean values followed by the same superscript in each column do not differ significantly at P≤0.05 level by DMRT

AIA: Actinomycetes Isolation Agar media, SCA: starch Casein Agar media, HVA: Humic Acid Vitamin Agar media, ISP: International *Streptomyces* Project -4 media, KA: Kuster's Agar media

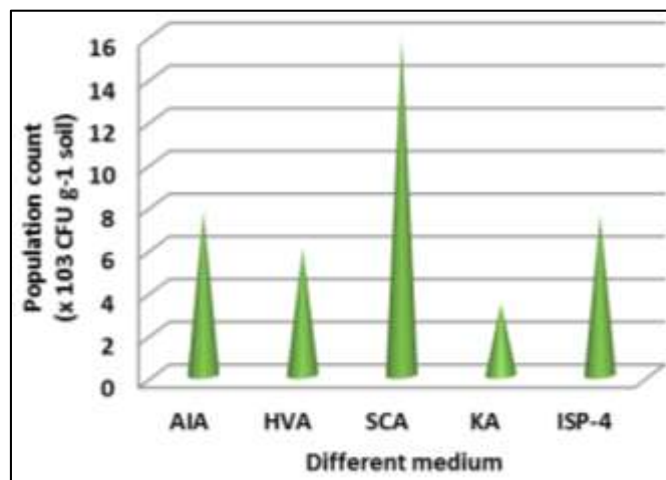

Fig 1: Enumeration of actinobacteria (No. x 10^3 CFU g^{-1} soil) on different growth Media

AIA: Actinobacteria Isolation Agar media, SCA: starch Casein Agar media, HVA: Humic Acid Vitamin Agar media, ISP: International *Streptomyces* Project -4 media, KA: Kuster's Agar media

Table 3a: Biochemical characteristics of actinobacterial isolates

Sl. No.	Actinobacterial isolates	Casein hydrolysis	Melanin production	Tyrosine activity	N-free agar medium	Gelatin hydrolysis	H_2S production
1	UASBA1	+	-	-	+	-	-
2	UASBA2	-	-	-	-	-	+
3	UASBA3	-	+	-	-	-	-
4	UASBA5	-	-	-	+	-	+
5	UASBA6	-	-	-	-	-	+
6	UASBA7	-	+	+	-	-	+
7	UASBA12	-	-	-	-	-	+
8	UASBA13	-	-	+	-	-	+
9	UASBA15	-	-	-	-	-	-
10	UASBA18	-	+	+	+	+	+
11	UASBA21	+	-	+	-	-	+
12	UASBA22	+	-	-	+	-	-
13	UASBA24	+	-	-	+	-	-
14	UASBA25	-	-	-	-	-	-
15	UASBA26	-	-	-	+	+	-
16	UASBA27	+	-	-	+	-	-
17	UASBA28	-	-	-	+	-	+
18	UASBA30	-	+	-	-	-	+
19	UASBA31	-	-	+	-	-	-
20	UASBA32	-	-	-	+	-	+
21	UASBA34	+	-	+	-	-	+
22	UASBA35	-	-	-	-	-	+
23	UASBA36	+	-	+	-	-	-
24	UASBA39	-	-	-	-	+	-
25	UASBA46	+	+	+	+	+	+
26	UASBA47	+	-	-	-	-	+
27	UASBA49	-	-	-	-	-	-
28	UASBA50	-	+	+	+	-	-
29	UASBA54	-	-	-	-	-	+
30	UASBA60	+	-	-	+	-	-
31	UASBA62	+	-	+	-	+	-
32	UASBA63	+	-	-	-	-	-
33	UASBA65	-	-	-	-	-	+
34	UASBA66	-	-	-	-	-	-
35	UASBA67	-	-	-	+	+	+
36	UASBA72	-	+	+	-	-	+
37	UASBA74	-	-	-	-	+	-
38	UASBA75	-	-	-	+	-	+
39	UASBA76	-	-	-	-	-	-
40	UASBA77	-	+	-	-	-	-

Note: +: Positive, -: Negative

Table 3b: Biochemical characteristics of actinobacterial isolates

Sl. No.	Actinobacterial isolates	Indole test	Methyl red test	Voges proskauer test	HCN production	Urease production	Citrate utilization
1	UASBA1	+	-	-	-	-	-
2	UASBA2	-	-	-	-	-	-
3	UASBA3	+	+	-	-	-	-
4	UASBA5	+	+	-	-	-	+
5	UASBA6	+	-	-	-	-	-
6	UASBA7	-	-	-	-	-	-
7	UASBA12	+	-	-	-	-	+
8	UASBA13	+	+	-	-	-	-
9	UASBA15	-	-	-	-	+	-
10	UASBA18	+	-	-	-	+	-
11	UASBA21	-	-	-	-	-	-
12	UASBA22	+	-	+	-	+	+
13	UASBA24	+	-	-	-	-	-
14	UASBA25	+	-	-	-	-	-
15	UASBA26	+	-	-	-	+	+
16	UASBA27	+	-	-	-	-	-
17	UASBA28	+	-	-	-	-	-
18	UASBA30	-	-	-	-	-	-
19	UASBA31	-	-	-	-	-	-
20	UASBA32	-	-	+	-	+	+
21	UASBA34	+	-	-	-	+	-
22	UASBA35	+	-	-	-	-	-
23	UASBA36	+	-	-	-	-	-
24	UASBA39	+	-	+	-	+	-
25	UASBA46	+	-	+	-	+	+
26	UASBA47	-	-	-	-	-	-
27	UASBA49	-	-	-	-	-	-
28	UASBA50	+	-	+	-	+	+
29	UASBA54	-	-	-	-	-	-
30	UASBA60	+	-	-	-	-	-
31	UASBA62	+	-	-	-	+	-
32	UASBA63	-	-	-	-	-	-
33	UASBA65	-	-	-	-	-	-
34	UASBA66	-	-	-	-	-	-
35	UASBA67	+	-	-	-	-	-
36	UASBA72	-	-	-	-	-	-
37	UASBA74	+	-	-	-	-	-
38	UASBA75	-	-	-	-	-	-
39	UASBA76	-	-	-	-	-	-
40	UASBA77	-	-	-	-	-	-

Note: +: Positive, -: Negative

Among the 40 actinobacterial isolates isolated from the different rhizosphere soil samples of legume crop, cowpea was rich in the actinobacteria population and starch casein agar was found as to be the best medium for the enumeration of actinobacteria. All the actinobacterial isolates were Gram positive and non-acid fast.

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