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Isolation and screening of plant growth promoting actinomycetes from rhizosphere of some forest medicinal plants

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Abstract : Rhizosphere is the area of intense microbiological activity. Plant growth promoting rhizomicroflora inhabit rhizosphere of plants, enhance plant growth production and release of metabolites and also inhibit soil borne plant pathogens. In the present study, a total of 62 actinomycetal isolates were obtained from the rhizosphere of some forest medicinal plants viz, *Calycopteris floribunda*(CFB), *Maeruga oblongifolia* (MO), Lantana camara (LC), *Zingiber officinale* (ZO) and *Schleichera oleosa* (SO) grown in the Pakhal forest, Pakhal wild life sanctuary, Warangal (Dt), Telangana State, India. All the isolates were screened for their plant growth promoting activities viz, Ammonia production, IAA production, HCN production and phosphate solubilization. The results showed that the actinomycetal isolates differed in the levels of PGP activities. The range of percentage (%) of positive isolates for each of PGP activities varied greatly: 47(75%) isolates showed Ammonia production, 45 (72%) isolates for indole acetic acid (IAA) production, 32 (51%) for hydrogen cyanide (HCN) production and 18 (29%) for phosphate solubilization. These results demonstrate the biotechnological potential of these microorganisms. Therefore, the present study suggests that these plant growth promoting actinomycetes (PGPA) may be used as biofertilizers to enhance the growth and productivity of commercially important medicinal plants.
Keywords : Medicinal plants, Actinomycetes, PGP activities, PGP substances.

Introduction

Actinomycetes have been and remain the most fruitful source of microorganisms for all types of bioactive metabolites, including agroactive type. Over one thousand secondary metabolites from actinomycetes were discovered during 1988-1992. Most of these compounds are produced by various species of the genus *Streptomyces*. In fact, about 60% of the new insecticides and herbicides reported in the past 5 years originate from Streptomyces[1]. It is also estimated that as many as three-quarters of all streptomycete species are capable of antibiotic production[2]. Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, b-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, anti-tumor and immunosuppressive activities[3].

Actinomycetes can promote plant growth by producing promoters such as indole-3-acetic acid (IAA) to help growth of roots or produce siderophores to improve nutrient uptake[4]. However, the rate of discovery of new secondary metabolites has been decreasing, so the discovery of actinomycetes from several sources increases the chance for the discovery of new secondary metabolites[5]. Active actinomycetes may be found in medicinal plant root rhizosphere soils and may have the ability to produce new inhibitory compounds. In

attempts to develop commercial biocontrol and plant growth promoting products using rhizobacteria, it is important to recognize the specific challenges they present. To begin with, the interaction between PGPR species and their plant symbionts appears to be specific, even within a crop or cultivar [6,7,8,9]. While a rhizobacterium screened for growth promotion may reveal positive effects on one crop, it may have no effect, or even retard growth of another crop [10, 11].

Pakhal forest is situated around the pakhal lake contains a wide variety of flora and fauna. The types of forests are dry deciduous and moist deciduous mixed forests with a few evergreen species. The soil is highly rich in organic matter and suitable for the growth of microorganisms. Although there are a number of reports on distribution and traditional uses of medicinal plants from these early human inhabitated areas, data on their microbial resources are scarce, imprecise, and not well documented. Due to uniqueness, large geographic variation, different soil types and the contents of this forest, it is quite likely that there is vast distribution of antibiotic producing actinomycetes in this environment. The present study was carried out to screen the most assured plant growth promoting actinomycetes from these unexplored soils rhizosphere of some medicinal plants.

Materials and Methods

Soil sampling and pretreatment

Soil samples were collected from different medicinal plants rhizosphere i.e, *Calycopteris floribunda* (CFB), *Maeruga oblongifolia* (MO), Lantana camara (LC), *Zingiber officinale* (ZO) and *Schleichera oleosa* (SO) grown in the pakhal forest, Warangal (Dt), Telangana State, India. Each collection was made from 6-12 inches depth of the surface of ground. These samples were placed in sterile poly bags, sealed tightly, and transported immediately to the laboratory. These soil samples were air-dried for 3-4 h at 45°C, cooled to room temperature and used for isolation purpose [12].

Isolation of pure culture of actinomycetes

Sixty two actinomycete strains were isolated as pure culture by using standard microbiological method [13]. One gram of dried soil was suspended in 99 ml sterile water and serially diluted in sterile water up to 10^{-7}. An aliquot of 0.1 ml of each dilution was taken and spread evenly over the surface of actinomycete isolation agar (AIA) medium (Actinomycetes isolation agar: K_2HPO_4-0.5 g; $MgSO_4.7H_2O$-0.1g; $FeSO_4.7H_2O$-1.0mg; sodium caseinate-2g; asparagines-0.1 g; sodium propionate-4g; glycerol-5g; agar-20g; distilled water- 1000 mL) [14], SCA (Starch Casein Agar: K_2HPO_4-2g, NaCl-2g, $MgSO_4.7H_2O$-0.05g, $CaCO_3$-0.02g, $FeSO_4.7H_2O$-0.01, soluble starch-10g; KNO_3- 2g; casein-0.3g; agar-20g; distilled water-1000mL), YEME (Yeast Extract Malt extract Agar: yeast extract-4g, Malt extract-10g ,glucose-4g, agar-20g; distilled water-1000mL) chitinase medium (Colloidal chitin-5g, yeast extract-0.5g, $(NH_4)_2So_4$-1g, $MgSo_4$-0.3g, KH_2PO_4-1.36g, agar-20g, distilled water-1000mL) supplemented with cyclohexamide (50µg/ml) and nystatin (50µg/ml) [15]. The dilutions were plated in duplicate. Plates were incubated at 30°C for 10 days to allow for spore formation. After incubation, dry, powdery and small colonies with extremely slow growth were counted and selected randomly from mixed plate culture and transferred by streaking to a new plate with the same culture medium for purification. It is possible that actinobacterial colonies belonging to different families that do not show these characteristics. The pure colonies were transferred to plates containing ISP2 culture medium (glucose-10g, malt extract-5g and yeast extract-5g with the pH adjusted to 7.2, agar-20g, distilled water-1000mL), and incubated at 28°C for one week. Subsequently, each isolate was stored in 40% glycerol for long time preservation. The isolates were replicated every 3 months.

Screening of Actinomycetes for PGP activities

All the isolates were screened for plant growth promoting activities i.e. Ammonia production, IAA production, HCN production and phosphate solubilization.

Ammonia production

The production of ammonia was determined by the method of [16]. The bacterial isolates were grown in peptone water for 7 d at 30°C. The presence of brown colour to deep yellow colour and on adding Nessler's reagent to the broth indicated maximum production of ammonia.

IAA production

The production of IAA was determined by the method of Gordon and Weber [17] by using the liquid SCA broth medium amended with tryptophan. Actinomycetal cultures were grown for 7d at 30°C on a rotary shaker. Fully grown cultures were centrifuged at 10,000 rpm for 15 min. The supernatant (2ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of Salkowski reagent (1ml of 0.5M $FeCl_3$ in 50ml of 35% $HClO_4$). Development of pink colour indicated the presence of IAA. Optical density was measured at 530nm by using a spectrophotometer.

HCN production

All the isolates were screened for the production of hydrogen cyanide by adopting the method of the Bakker and Schipper [18]. For this King's B medium was amended with glycine at 4.4 g/l and bacteria were streaked on agar plate. Whatman filter paper No.1 (9 cm in diam) soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the lid of the plate. Plates were sealed with parafilm and incubated at 30°C for 7d. The change in colour of filter paper from deep yellow to orange and finally to orange brown to dark brown indicated the production of HCN.

Phosphate solubilization

Phosphate solubilization was determined[19] by inoculating the isolates onto Pikovskya agar medium and subsequent incubation of plates. After 7days, plates were observed for clearing zones around the bacterial growth.

Results and Discussion

Isolation of PGPR from rhizosphere of medicinal plants.

For isolation and quantification of the actinobacteria in rhizosphere soil of forests, the most adequate dilution of a ten-fold dilution series was 10^{-3}, resulting an average of ten colonies per plate. However, the number of contaminants was greater, when comparing with the 10^{-4} dilution. The number of actinobacteria detected in this study is in line with Crawford et al. (1993) who reported a greater amount of actinobacteria in rhizosphere in comparison with surrounding soil. Media containing antibiotics were used in our study because there are reports about their action also against the actinobacteria themselves [21]. A total of 62 actinomycetal strains were isolated from the rhizosphere of *Calycopteris floribunda, Lantanus camera, Slyschera species, Zingiber officinale* and *Maeruga oblongifolia*. Among the 62 isolates, 22 actinomycetal strains were isolated from *Calycopteris floribunda,* 7 strains from *Lantanus camera,* 21 from *Schleichera oleosa,* 10 from *Zingiber officinale* and 2 from *Maeruga oblongifolia.*

Characterization of PGPR

All the isolates were screened for their plant growth promoting activities viz., Ammonia production, indole acetic acid (IAA) production, phosphate solubilization, and HCN production. The results showed that not all the isolates possessed all the PGP activities (Table 1). The range of percentage of positive isolates for each of PGP activities varied greatly. Among the 62 isolates, 47 isolates were showing Ammonia production, 45 isolates were showing IAA production, 32 for HCN and 18 for phosphate solubilization (Fig.3). Among the 62 actinomycetal strains 75% of strains were positive for ammonia production while 72% for IAA, 51% for HCN and 29% of strains showed phosphate solubilization (Fig.2). Although much higher values of PGP activities also have been found. Microorganisms that produce auxins, as indole-acetic acid, are widely distributed in soil [22, 23]. Sousa et al. [24] already stated that actinobacteria have a good potential as indole-acetic acid producers in Brazil. These auxins stimulate the growth and development of plants. Therefore, they may be useful in the management of forest species mainly in nurseries or during transplantation to the field.

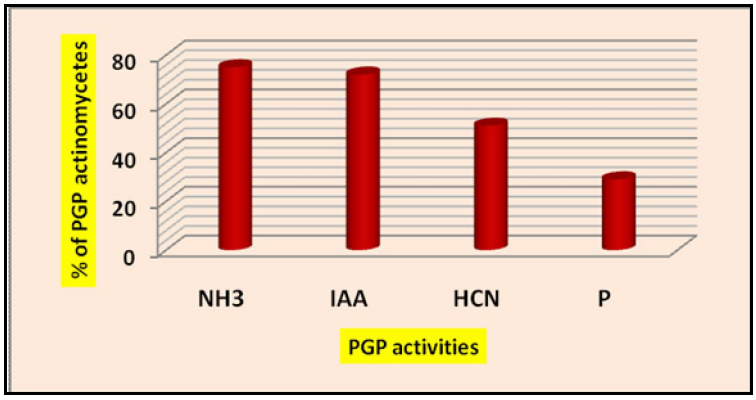

Figure 2. Percentage (%) of actinomycetes showing PGP activities

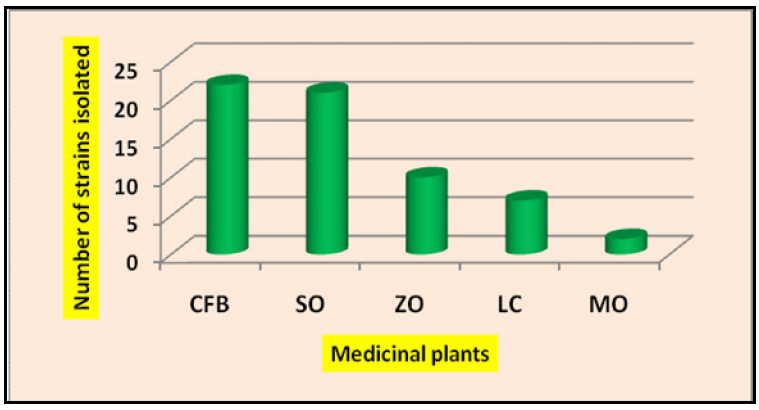

Figure 3. Number of strains isolated from different medicinal plants

Table 1. PGP activities of Actinomycetes from the rhizosphere of medicinal plants

S.No.	Isolate No.	Colour of the colony	NH_3 Production	IAA Production µg/ml	HCN Production	PO_4 Solubilization
1	CFB-1	Gray	+++	0.11	+	-
2	CFB-2	White	+	0.08	-	-
3	CFB-3	Brownish	+++	0.21	+	-
4	CFB-4	Brown	+++	0.61	+++	-
5	CFB-5	Gray	+++	0.15	-	
6	CFB-6	White	+++	0.37	+	++
7	CFB-7	Cream-white	+	0.00	+	-
8	CFB-8	Thick Brown	+++	0.51	-	-
9	CFB-9	Brown	+++	0.22	++	+
10	CFB-10	Brown	+++	0.51	+++	-
11	CFB-11	Gray	+++	0.90	+	-
12	CFB-12	Gray	-	0.12	+++	+++
13	CFB-13	Cream-white	+	0.000	-	-
14	CFB-14	Brown	+++	0.000	-	-
15	CFB-15	Gray	-	0.06	+	-
16	CFB-16	white	-	0.025	+	-
17	CFB-17	Violet	-	0.24	-	-
18	CFB-18	Pink-white	-	0.00	-	++
19	CFB-19	white	++	0.016	+++	-
20	CFB-20	Cream-white	++	0.047	++	-
21	CFB-21	Gray	+++	0.000	-	++
22	CFB-22	white	+++	0.20	-	+

23	MO-1	white	++	0.016	-	-
24	MO-2	White	++	0.06	-	-
25	LC-1	Cream- white	+	0.107	-	-
26	LC-2	Thick Gray	++	0.008	-	-
27	LC-3	Gray	++	0.213	++	-
28	LC-4	Cream white	+++	0.00	+++	-
29	LC-5	Greenish white	+++	1.045	-	+
30	LC-6	Cream white	-	0.50	-	+
31	LC-7	Gray	+++	0.943	-	++
32	ZO-1	Greenish Gray	+++	0.00	-	-
33	ZO-2	Black-Gray	++	0.04	+	-
34	ZO-3	Gray	-	0.10	++	-
35	ZO-4	Gray	+	0.00	+	-
36	ZO-5	Gray	++	0.50	+	+
37	ZO-6	Greenish cream	+++	0.12	+	+
38	ZO-7	Greenish cream	+	0.00	-	-
39	ZO-8	white	++	0.23	-	-
40	ZO-9	white	+	1.23	+++	-
41	ZO-10	white	-	0.14	-	-
42	SO-1	Violet	+	2.00	++	-
43	SO-2	Baby pink	-	2.50	-	-
44	SO-3	Gray	-	0.12	-	-
45	SO-4	Gray	++	0.15	-	-
46	SO-5	Gray	++	0.10	++	-
47	SO-6	Gray	++	0.00	+++	+
48	SO-7	Gray	+++	0.24	+++	-
49	SO-8	White	+++	0.22	++	-
50	SO-9	White	+++	0.05	-	-
51	SO-10	Gray	++	0.00	-	+
52	SO-11	Brown	++	0.24	++	-
53	SO-12	Gray	-	0.06	-	++
54	SO-13	Gray	+++	0.00	-	-
55	SO-14	Gray	-	0.07	-	++
56	SO-15	Gray	-	0.12	+	++
57	SO-16	White	++	0.00	-	-
58	SO-17	Gray	++	0.07	+	+++
59	SO-18	White	-	0.09	+	-
60	SO-19	White	-	0.08	++	-
61	SO-20	Gray	+	0.00	+	+
62	SO-21	Gray	+	0.00	-	-

- = No production; + = weak producer; ++ = medium producer; +++ = high producer; CFB= Calycopteris floribunda, MO= Maeruga oblongifolia, LC= Lantana camara, ZO=Zingiber officinale, SO=Schleichera oleosa

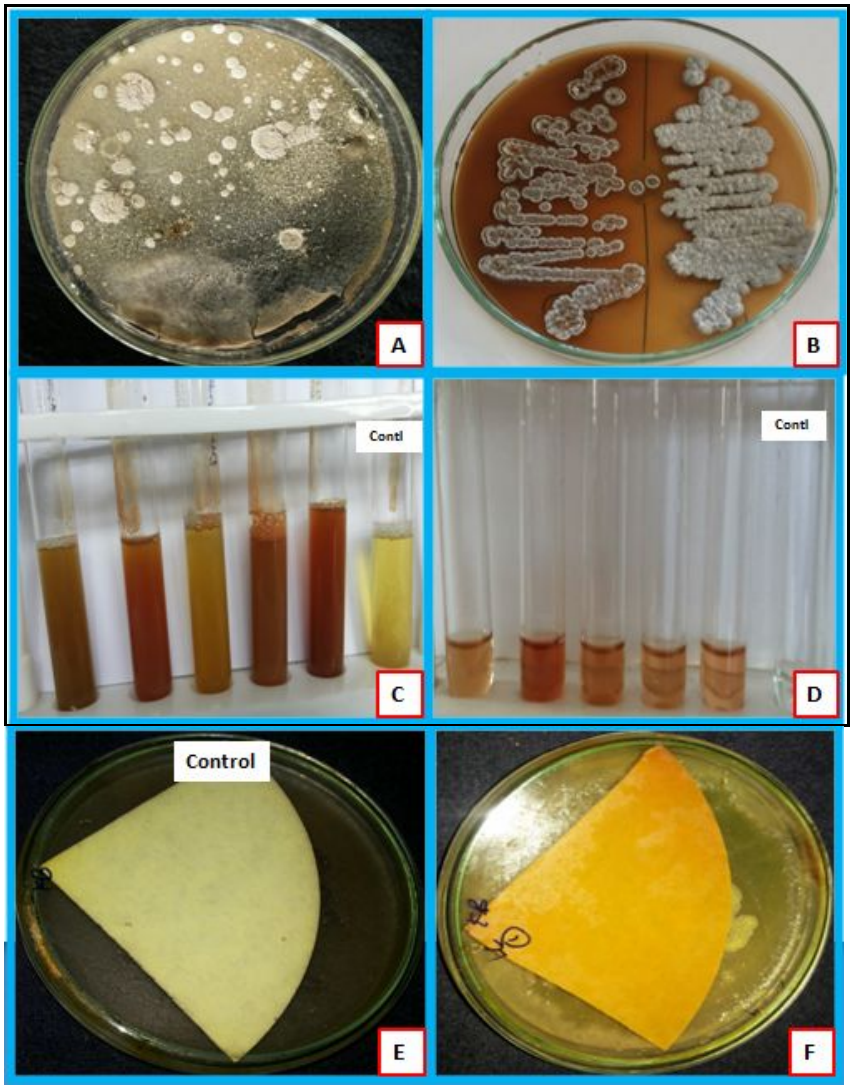

Figure 1. Characterization of plant growth promoting actinomycetes (PGPA) from rhizosphere of medicinal plants: A =isolation of actinomycetes; B =growth of colony on YEME media, C=ammonia production, D=IAA production, E, F=HCN production: control, production of HCN by PGPA.

There is a growing interest in the possibility of using microorganisms in the rhizosphere in favour of plant growth and productivity. Some studies on rhizosphere actinomycetes have revealed that there is positive rhizosphere effect and that they have an antagonistic effect on fungal root pathogens [25]. Our knowledge on the natural flora of actinomycetes in rhizosphere is however very poor (Fig.1A,B).

The results showed the actinomycetal strains produced high levels of PGP substances in different traits. Almost every isolate showed at least two PGP activities, and some strains showed high PGP activities. 21 (33.8%) strains showed high, 16 (25%) medium, 10 (16%) strains showed weak production of ammonia (Fig.1C) and only 15 (24%) strains are failed to show ammonia production. The indole acetic acid (IAA) production range from minimum 0.040 µg/ml to 2.50 µg/ml. Only 2 (3%) strains showed high indole acetic acid (IAA) production, 2 (3%) are medium, 41 (66%) strains showed weak production of indole acetic acid. The maximum IAA production was recorded by SO-2 (2.50µg/ml) (Fig.1D). The maximum HCN production (Fig.1F) was recorded by 8 (12%) isolates, 9 (14%) are medium, 15 (24%) are weak producers and 30 (48%) strains are failed to produce HCN. However only 2 (3%) isolates showed high Phosphate solubilizin ability 7 (11%) are medium, 9 (14%) are weak producers and remaining 44 (71%) strains are failed to show clear zone around the colony. Simililar studies are carried out by Sutthinan Khamna et al.[26] and Rafael Leandro Figueiredo de Vasconcellos[27].

These results demonstrate the biotechnological potential of these microorganisms. Thus the rhizosphere isolates from medicinal plants are able to solubilize phosphate, produce hydrolytic enzymes, phytohormones

and have multiple PGP activities, and plant disease suppression. Such characteristics confirm that these rhizosphere microorganisms are PGP microbes. These isolates can be further used as bioinoculum and can be exploited for the synthesis of numerous metabolites which can be used commercially. Further trials and in depth studies are required to check whether these PGP actinomycetes have any influence on the production of environment specific active principle in many other medicinal plants.

Conclusion

In conclusion, our results suggest that simultaneous screening of actinomycetes for plant growth promoting activities is a good tool to select effective PGP actinomycetes for biofertilizer development. Results suggest that PGP isolates which are able to produce multiple PGP activities like IAA production, ammonia and solubilize the phosphate, may improve the growth of plants. The prospective species could be recovered from enrichment methods. The use of such PGP actinomycetes singly or in consortium can act as efficient bioinoculants which may be an approach to reduce the usage of chemical fertilizers and pesticides for sustainable cultivation of medicinal plants.

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