Isolation And Purification Of Plant Growth Promoting Rhizobacteria (Pgpr) From The Rhizosphere Of *Acorus Calamus* Grown Soil

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ABSTRACT.

Among the 10 bacterial isolates were obtained from Acorus calamus rhizospheric soil of Melaiyar and Nagapattinam districts in Tamil Nadu. All the isolates were identified as Azospirillum spp., Bacillus spp., Pseudomonas spp., and Azotobacter spp. These bacterial strains were tested on morphological, biochemical and screened for their direct growth promoting activities (IAA production, production of Ammonia and Phosphate solubilization) and indirect growth promoting activities (HCN production, Siderophore production). The results obtained showed that among the 10 isolates of Melaiyar and Nagapattinam districts (M-M15) of ranged from (4.10 - 5.66 and 4.40 - 7.80) of Azospirillum spp., (3.00 – 6.00 and 3.20 – 6,.30) of Bacillus spp., (5.00 – 8.40 and 5.10 - 8.20) of Pseudomonas spp., and (3.00 - 6.00 and 3.30 - 6.30) of Azotobacter spp. The IAA production of Pseudomonas spp. (94%), Azospirillum spp (80%), Azotobacter spp. (65%) and Bacillus spp. (40%). Ammonium production of the isolates, Bacillus spp. (96%), Pseudomonas spp. (92%), Azospirillum spp. (65%) and Azotobacter spp. (55%). The siderophore and HCN production produced by all the isolates make it suitable for further investigation of pot and field trials by Acorus calamus cultivation

KEYWORDS:

Acorus calamus, PGPR

INTRODUCTION:

Acorus calamus *(A. calamus)* L. also known as sweet flag is a native plant of India. It is commonly known as Bach Tamilnadu in India. It is aquatic perennial, aromatic herb with creeping rhizomes. It exhibits polyploidy. This plant belongs to araceae family and has been used in the Indian and chinese system of medicine for hundreds of years to cure disease especially present like effect of *A. calamus* rhizomes using reported experimented. The different parts of names in Bach (Hindi), vasambu (Tamil), Baje (Kanada), and vasa (Telugu). The medicinal plants are the richest bioresource of drugs for traditional systems of medicine; therefore man has been using plant extracts to protect himself against several disease and also to improve his health and lifestyle (Parekh *et al.*, 2007).

In aurvedic medicine, It is used low the treatment of skin eruptions, epilepsy, mental ailments, chronic diarrhea, dysentery, rheumatic pains, neuralgia, cancer dyspepsia and bronchial catar intermittent fever, (Sabitha *et al.*, 2003) in addition its plant extract is mainly used for various pharmacological actives like.

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at rhizome and in association with rhizomes, with can improve the extract or quality of plant directly and indirectly. In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Bacillus,* have reported to enhance plant growth (Kloepper *et al.*, 1989); Okon an Laban dera – Gonzolez., 1994; Glick, 1995). The promotion by PGPR either providing the plant with a growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment.

MATERIALS AND METHODS

In the experimental area of the potanical garden of the Annamalai University of Chidambaram, there was a formerly planted *ex situ* population the individuals started to grow fast. Later on several competitors in the filed. Although the competition level was high, there was large calamus grown in plant.

Isolation of Rhizobacteria:

The bacteria using for testing the bacterial property of *A.calamus.* The ten location of Rhizospheric soil sample were collected from communally grown *A. calamus* from melaiyur in Nagapattinam districts of Tamil Nadu. All the bacterial strains were isolated on their respective media, *Azospirillum* was on Nitrogen free malic acid medium (NFb), Determiner and Day (1957), *Azotobacter* an Waksman base No.77 Medium (Allen, 1953), *Bacillus* (Phosphobacteria) on Pikovskaya's Agar medium (Gaur, 1990), Pseudomonas on King's B medium (King's *et al.*, 1954). The bacterial culture were maintained on the respective stands. The bacterial isolates were designated as melaiyur (M_1-M_{10}) in Nagapattinam district of Tamil Nadu and species level identification of all rhizobacteria of melaiyur. *Azospirillum* (MAzs-1 to MAzs-10), *Azotobacter* (MA_{zt}-1 to MA_{zt}-10), *Bacillus* [MB-1 to MB-10], *Pseudomonas* (MPf-1 to MPf-10).

Biochemical characterization of PGPR strains

Selected thirty isolates of *Azospirillum, Bacillus, Pseudomonas* and *Azotobacter* were biochemically carried out. The following biochemical test were carried out separately for *Azospirillum* (pellicle formation, cell shape, motility, gram reaction, acid production from glucose, different carbon sources, malate,succinate, lactose,

mannitol, a-ketoglutarate, biotin requirement, nitrate reductase, nitrite reductase activity), *Bacillus* (gram reaction, motility, spore staining, acid production, hydrolysis of starch, hydrolysis of gelatin, casein hydrolysis, catalase test, oxidase test, indole test, methyl test, urease test, VP test, utilization of citrate), *Pseudomonas* (gram reaction, motility, starch hydrolysis, hydrolysis of gelatin, egg yolk reaction, pigment production, casein hydrolysis, catalase test, oxidase test, indole test, methyl red, citrate utilization test, H_2S production), and *Azotobacter* (gram reaction, motility, pigmentation, catalase test, oxidase test, indole test, utilization of citrate, utilization of carbon sources, etc.,) as per the standard methods (Cappuccino and Sherman, 1992).

***In vitro* screening of bacterial isolates for their plant growth promoting (PGP) activities for indole acetic acid (IAA) production**

IAA production was detected by the modified method as described by Brick *et al.* (1991). Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986) at 100% concentration of tryptophan (100µg/ml). Bacterial cultures were grown for 72 h (*Azotobacter* and *Azospirillum)* and 48 h *(Pseudomonas* and *Bacillus)* on their respective media at 25±2°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production. Optical density was taken at 530 nm with the help of spectrophotometer Spectronic 20 D^+. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10-100 µg/ml.

Production of NH_3

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 25±2°C. Nessler's reagent (0.5, ml) was added in each tube. Development of blue to light yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Production of HCN

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine/1 and bacteria were streaked on modified agar plate. A Whatman filter paper No. 1 soaked in 2% sodium carbonate in 0.5%) picric acid solution was placed in the top of

the plate. Plates were sealed with parafilm and incubated at 25 ± 2°C for 4 days. Development of orange to red colour indicated HCN production.

Siderophore production

Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (l0µl of 10^6CFU/ml) and incubated at 25±2ºC_ for 48-72 h. Development of golden yellow-orange halo around the growth was considered as positive for siderophore production.

phosphate solubilization by test bacteria

All isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by Gaur (1990). Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was made as described by King (1932). Briefly, the test isolates were inoculated in 25 ml Pikovskaya's broth and incubated for 4 days at 25 ± 2°C. The bacterial cultures were centrifuged at 15,000 rpm for 30min. Supernatant (1ml) was mixed with 10 ml of chloromolibidic acid and the volume was made up to 45 ml with distilled water. Cholorostannous acid (0.25 ml) was added and the volume was made up to 50 ml with distilled water. The absorbance of the developing blue colour was read at 600 nm. The amount of soluble phosphorus was detected from the standard curve of KH_2PO_4.

RESULTS

The plant Growth promoting rhizobacteria population in Rhizophere of *A.calamus* is given in Table.1. The PGPR population (CFU g^{-1} of oven dry soil) Melaiyur in Nagapattinam districts of ranged from (4.44-10.44 x 10^6) *Azospirillum* spp., (3.22-8.88 x 10^6) *Azotobacter* spp., (4.22-8.66 x 10^6) *Bacillus* spp., (3.00-12.00 x 10^6 *Pseudomonas* spp).

Plant growth promoting traits of test isolates

In the present investigation 10 isolates of *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp., and *Azotobacter* spp. Screening results of PGP traits are depicted in (Fig.1 & Fig.2). IAA production was shown in all the isolates of *Pseudomonas* (87%), followed by *Azospirillum* (80%), *Azotobacter* (65%) and *Bacillus* (40%).

Ammonia production was detected in (98%) of isolates of *Bacillus* followed by *Pseudomonas* 94%. *Azospirillum* (65%) and *Azotobacter* (55%). Phosphate solubilzation was detected in 83% of isolates of *Bacillus* followed by *Azotobacter* (68.47%), *Pseudomonas* (60.56%) and *Azotobacter* (68.47%), *Pseudomonas* (60.56%) and *Azospirillum* (55%). Production of siderophore was detected less frequently than other PGP characteristics. The isolates of *Pseudomonas* spp. were strong siderophore producers (18.22%) followed by *Azospirillum* spp. (16.22%). The production of HCN was detected for all cultures in less frequently. The *Pseudomonas* spp. were maximum produced (60%), followed by *Bacillus* spp. (45%), *Azopirillum* spp. (20%) and *Azotobacter* spp. (10%).

Quantitative assay of IAA production by PGPR strains

A total of 10 isolates of *Azopirillum* spp, *Azotobacter* spp, *Bacillus* spp, and *Pseudomonas* spp. were tested for the quantitative estimation of IAA in the presence of 100% of Tryptophan concentration.

Table – 1

Plant growth promoting rhizobacterial total population of *Acorus calamus* from commercially grown area.

Population (x10^6 CFUg-1 of day soil sample)

S.No	Azosprillum MA_{zs}-1 to MA_{zs}-10	Azotobacter MA_{zt}-1 to MA_{zt}-10	Bacillus MB-1 to MB-10	Pseudomonas MPf-1 to MPf -10
Malaiyur				
M1	10.44	8.88	8.66	12.00
M2	8.00	7.44	7.66	10.22
M3	5.66	7.66	6.22	11.33
M4	8.88	6.55	6.33	9.66
M5	6.77	7.22	8.22	9.22
M6	9.00	7.88	6.33	8.33
M7	7.33	6.22	5.44	6.66
M8	5.22	3.44	4.66	4.88
M9	5.00	5.33	4.88	5.00
M10	4.44	3.22	4.22	3.00

Isolation and Purification of Plant Growth Promoting Rhizobacteria in Microorganisms

Fig. 1 *Azospirillum*

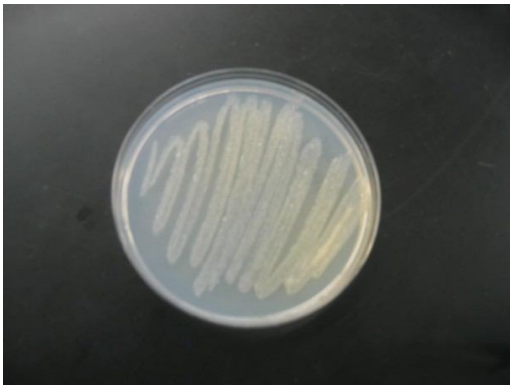

Fig. 2 *Azotobacter*

Fig. 3 *Bacillus*

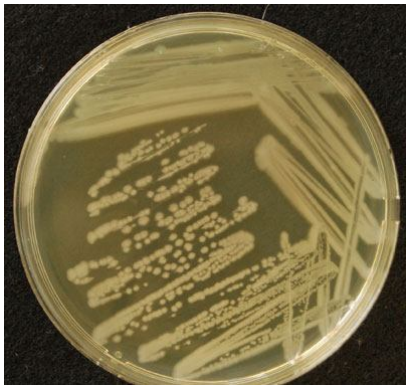

Fig. 4 *Pseudomonas*

DISCUSSION

Plant rhizosphere is various types of soil microorganisms due to rich Nutrients availability. It has been diazotrophic bacteria like *Bacillus, Azotobacter* and *Azospirillum* enhanced the plant growth as a result of ability to base nitrogen. The plant growth promoting harmones in the rhizospher and other (PGP) activities. (Kloepper *et al.,* 1988); Arshad and Frankenberger 1993 and Bashan 2005).

Out of 10 of Melaiyur in Nagappattinam belonging to 5 isolates fo *Azospirillum lipoferum*, 5 districts isolates of *Bacillus megatreium* and 5 isolates of *Pseudomonas fluorescens*, and 5 isolates of *Azotobacter chroococcum*, were screened *in vitro* for PGP activities. The potential of *Pseudomonas* strains to produce indole acetic acid under *in vitro* condition was reported. All the 10 isolates obtained in the present study were able to produce IAA. The IAA production was detected in all the test isolates of *Pseudomonas fluorescens* (87%), *Azospirillum lipoferum* (80%) and *Azotobacter chroococcum* (65%), followed by *Bacillus megatreium* (40%), High level of IAA production by *Pseudomonas* was recorded by other workers (Xie *et al.*, 1996). Our findings of IAA in *Azotobacter* isolates and in agreement with other workers (Gonzalez-Lopez *et al.*, 1986; Jagnow, 1987; Nieto and Frankenberger, 1989).

On the basis of preliminary screening, quantitative analysis of IAA production was made on 10 isolates Melaiyur in Nagapattinam *Azospirillum* spp, *Azotobacter* spp, *Bacillus* spp and *Pseudomonas* spp. There was an increase in the level of IAA with the concentration of tryptophan (100µg/ml). Similar trend of IAA production with increasing concentration of tryptophan was also reported by Barazani and Friedman (2000). The maximum IAA production isolates of *Pseudomonas* spp. of (5.00-8.40 µg/ml and 5.10-8.20 µg/ml) followed by *Azospirillum* spp. of (4.10-5.66 µg/ml and 4.40-7.80 µg/ml), *Azotobacter* spp. (3.00-6.00 µg/ml and 3.30-6.30 µg/ml), and *Bacillus* spp. of (3.00-6.00 µg/ml and 3.20-6.40 µg/ml). Among the 10 PGPR isolates, *Pseudomonas* spp.

Table-2

General characteristics of Azospirillum isolates obtained from the rhizosphere soil of *Acorus calamus* grown area

No.	Name of the isolated	Subsurface pellicle formation semi-solid medium	Cell shape	Motility	Gram reaction	Acid production from glucose	Malate	Succinate	Lactose	Mannitol	Keto lutarate	Biotin requirement	Nitrite reductase activity	Nitrate redutase activity	species identification
Melaiyur															
1.	MAzs-1	+	Rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*
2.	MAzs-2	+	Rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*
3.	MAzs-3	+	Rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*

4.	MAzs-4	+	Curved rod	+	-ve	-	+	+	+	+	+	-	+	-	*A.brasilense*
5.	MAzs-5	+	Rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*
6.	MAzs-6	+	Rod	+	-ve	-	+	+	+	+	+	-	+	-	*A.brasilense*
7.	MAzs-7	+	Rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*
8.	MAzs-8	+	Rod	+	-ve	-	+	+	+	+	+	-	+	-	*A.brasilense*
9.	MAzs-9	+	Curved rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*
10.	MAzs-10	+	Curved rod	+	-ve	+	+	+	+	+	+	+	+	+	*A.lipoferum*

(+) showed positive growth, (-) showed No growth

Table-3
General characteristics of *Azotobacter* isolates obtained from the rhizosphere soil of *Acorus calamus* grown area

Sl. No.	Name of the isolated	Gram reaction	Motility	Pigments		Catalase	Oxidase test	Indole test	Methyl test	Citrate utilization test	Utilization of Different Carbon Source		species identification
				Water soluble	Water insoluble						Starch	Raffinose	
Melaiyur													
1.	MAzt-1	-ve	+	+	Brown to Blue	+	+	+	+	+	+	+	*Azotobacter chroococcum*
2.	MAzt-2	-ve	+	+	Brown to Blue	+	+	+	+	+	+	+	*chroococcum*
3.	MAzt-3	-ve	+	+	Brown to Blue	+	+	+	+	+	+	+	*A.chroococcum*
4.	MAzt-4	-ve	+	+	Pale color	+	+	+	+	+	+	+	*A.chroococcum*
5.	MAzt-5	-ve	+	+	Brown to Blue	+	+	+	+	+	+	+	*A.vinelandii*
6.	MAzt-6	-ve	-	+	Yellowish	-	+	+	+	+	-	-	*A.chroococcum*
7.	MAzt-7	-ve	-	+	Yellowish	-	+	+	+	+	-	-	*A.beijerinckii*
8.	MAzt-8	-ve	+	+	Brown to Blue	+	+	+	+	+	+	+	*A.chroococcum*
9.	MAzt-9	-ve	-	+	Yellowish	-	+	+	+	+	-	-	*A.beijerinckii*
10.	MAzt-10	-ve	+	+	Pale color	+	+	+	+	+	+	+	*A.vinelandii*

(+) showed positive growth, (-) showed No growth

Table-4
General characteristics of *Bacillus* isolates obtained from the rhizosphere soil of *Acorus calamus* grown area

No.	Name of isolate	Gram reaction	Motility	Spore standing	Acid production	hydrolysis of starch	Casein hydrolysis	Catalyses test	Oxidize test	Indole test	Methyl test	Unease test	VP test	Utilization of citrate	Species identification
Melaiyur															
1.	MB-1	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*Bacillus megaterium*
2.	MB-2	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*B.megaterium*
3.	MB-3	+ve	+	+	+	-	+	-	+	+	-	-	+	+	*B. polymax*
4.	MB-4	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*B.megaterium*
5.	MB-5	+ve	+	+	+	-	+	-	+	+	-	-	+	+	*B. subtillis*
6.	MB-6	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*B.cereus*
7.	MPB-7	+ve	+	+	+	-	+	-	+	+	-	-	+	+	*B. polymax*
8.	MB-8	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*B.megaterium*
9.	MB-9	+ve	+	+	+	-	+	-	+	+	-	-	-	-	*B.megaterium*
10.	MB-10	+ve	+	+	+	-	+	-	+	+	-	-	+	+	*B.megaterium*

(+) showed positive growth, (-) showed No growth

Table-5
General characteristics of *Pseudomonas* isolates obtained from the rhizosphere soil of *Acorus calamus* grown area

Sl. No.	Name of the isolated	Gram reaction	Motility	Starch hydrolysis	Hydrolysis of gelatin	Egg yolk reaction	Pigment production	Casein hydrolysis	Catalase test	Oxidase test	Indole test	Methyl test	Citrate utilization test	H_2S production	species identification
Melaiyur															
1.	MPf-1	-ve	+	-	+	-	+	+	+	+	-	-	+	-	*Pseudomonas*
2.	MPf-2	-ve	+	-	+	-	+	+	+	+	-	-	+	-	*fluorescens*
3.	MPf-3	-ve	+	-	+	-	+	+	+	+	-	-	+	-	*P.fluorescens*

4.	MPf-4	-ve	+	-	-	-	+	+	+	+	-	-	+	-	*P.fluorescens*
5.	MPf-5	-ve	+	-	+	-	+	+	+	+	-	-	+	-	*P.fluorescens*
6.	MPf-6	-ve	+	-	-	-	+	+	+	+	-	-	+	-	*P.putida*
7.	MPf-7	-ve	+	-	-	-	+	+	+	+	-	-	+	-	*P.striata*
8.	MPf-8	-ve	+	-	-	-	+	+	+	+	-	-	+	-	*P.striata*
9.	MPf-9	-ve	+	-	+	-	+	+	+	+	-	-	+	-	*P.fluorescens*
10.	MPf-10	-ve	+	-	-	-	+	+	+	+	-	-	+	-	*P.putida*

Production of indole acetic acid (IAA) by rhizobacterial isolates grown on their respective medium

	IAA production at 100% tryptophan concentration (µg/ml)			
No. of isolates	*Azosprillum MA_{zs}-1 to MA_{zs}-10*	Azotobacter MA_{zt}-1 to MA_{zt}-10	Bacillus MB-1 to MB-10	Pseudomonas MPf-1 to MPf -10
Melaiyur				
M1	6.66	6.00	5.73	7.40
M2	5.97	5.10	4.87	7.10
M3	6.44	5.30	5.36	6.83
M4	6.00	5.93	3.25	6.00
M5	3.88	4.77	3.77	6.13
M6	6.38	4.30	2.83	7.40
M7	5.00	5.96	4.00	7.00
M8	4.00	4.00	3.53	7.93
M9	4.67	4.87	5.20	6.88
M10	5.28	5.25	3.60	6.50

*For Azspirillum Nfb media, Bacillus Pikovskaya's media, Pseudomonas king's B media, Azotobacter Waksman base media.

The knowledge β-Asarone was the major constituent in the leaves, whereas acorenone was dominant in the rhizomes (Venskutonsis *et al.,* 2003. β-asarone (cis-isomer) and evgenol were also identified (Kindscher *et al.,* 1992). Previous reports have shown that the *A. calamus* is rich in diverse lerpenoids, generally regarded as its characteristics of components was soil sample isolated, instead the present investigation is a part of research work being carried out on the micro organisms present *Azospirillum, Azotobacter, Bacillus* and *Pseudomonas* plant grown in rhizosphers soil and environment factors.

The rhizospheres of *Acorus calamus* soil sample collected from to different locations of melaiyur in Nagapattinam district of Tamilnadu, were determined. The populations of *Azospirillum* ranged from (4.44-10.44 x 10^6), *Azotobacter* population ranged from (4.22-8366x10^6), *Pseudomonas* population ranged from 3.00-12 x 10^6) of soil followed by others. The similar report done by Govinda Rao *et al.,* (1987). Geetha (2003) and Karthikeyan *et al.,* (2008) . microbial population from various medicinal plants.

IAA than that of other isolates, which was followed by *Azospirillum* spp, *Azotobacter* spp. and *Bacillus* spp. The highest IAA production of *Pseudomonas fluorescens* (MPf-1) 8.00µg/ml and *Pseudomonas fluorescens* (MPf-1) 7.60 µg/ml followed by other isolates produced from melaiyur in Nagapattinam districts. The variation in the production of IAA by different isolates of PGPR and its related role on the plant growth promoting activity was earlier studies under in vitro conditions (Crozier and Arrude, 1988; Gopal, 2004). In addition to these traits, plant growth promoting rhizobacterial strains must be rhizospheric component, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil plant system under pot and field conditions.

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