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Siderophore producing Plant Growth Promoting Rhizobacteria and its improvement of Oil yielding crops

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Abstract: **Siderophores are small protein molecules that can easily bind to ferric iron. As a chelating agent they transport iron molecules inside the bacterial cell for various biochemical reaction. At present studies characterization of few siderophores producing bacteria from the soil samples collected from Salem district, Tamil Nadu. The siderophores production was assayed qualitatively and quantitatively through Chrome Azural S and the results showed positive for the strains PSA01 and PSA02 that grown in succinate medium. In pot culture studies *Sesamum indicum. L* showed significantly increase in the root length, shoot length, fresh weight, dry weight and total chlorophyll and carotenoids significantly increased in treated plant. The antagonist activity of the siderophore producing *Pseudomonas* spp against fungal pathogen *Fusarium oxysporum.* This result showed that *Pseudomonas* spp is a good producer of siderophore which can be usefully exploited as a biocontrol agent and also as plant growth promoter and increasing the Oil yielding crops.**

Keywords: **Biocontrol, chlorophyll, carotenoids, *Pseudomonas spp,* siderophore, *Sesamum indicum. L.***

1. INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) directly associate with plant roots and can exist within root tissue (as endophytes), on the surface of plant roots (the rhizoplane), or within the zone of soil specifically influenced by the root system (the rhizosphere). Characterized by their plant-growth promoting properties, PGPR are a diverse group of bacteria that produce a wide range of enzymes and metabolites, influence nutrient acquisition, modulate hormone levels, and ameliorate the negative impacts of biotic and abiotic stressors (Ngumbi and Kloepper 2016)

Pseudomonas are excellent candidates of PGPR and can produce hydrogen cyanide, siderophores, protease, antimicrobial compounds and possess phosphate solubilizing activity. (Noor *et al.,*2012). Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Many siderophore are non-ribosomal peptides. (Hu *et al*., 2011). At present nearly 500 siderophores are reported from selected microorganisms. A great variation is seen in siderophores structures from one species to another (Alli and vidhale *et al*., 2013).

Biological control has emerged as a very popular alternative because it offers a way of controlling pathogens that does not involve the use of chemicals. Siderophores (derived from the Greek meaning 'iron carriers') are low molecular weight (below 1000Da), ferric –ion-specific chelating agents produced by bacteria and fungi to compact low iron stress (Ngamau *et al*., 2014).

Sesame (*Sesamum indicum L.*) seed has rich source of fatty acids and antioxidants, so it is used as healthy and nutritive additive to prepare several foods. The yield of sesame has been affected by several abiotic and biotic factors. The seedlings emergence, plant growth and the yield of *sesame* are inhibited up to 40%, during the effect of destructive pathogens of *Fusarium oxysporum* and *Macrophomina phaseolina*, which cause wilt and charcoal rot disease (Kumar *et al.*, 2011).

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Siderophore producing PGPR play a vital role in Fe nutrition of plants and therefore in plant growth promotion leading to healthy plants, which are vital for increasing the crop and food yield. Siderophores of rhizobacteria provide iron nutrition to the plants and help in plant growth promotion. (Shaink *et al.,* 2014). They prevent the availability of iron to plant pathogens thereby restricting its growth, and this, help in biological control of phytopathogens.

2. MATERIALS AND METHODS

2.1. Isolation of soil microbes: Rhizosphere soils were collected from plant rhizosphere region Pagalpatti, Pannapatti. Poosaripatti (Omalur), Salem. TamilNadu. The collected soil sample was brought to the laboratory in sterile polythene bag under aseptic conditions the soil sample was air dried and subjected to the isolation of bacteria by spread plate technique.

2.2. Biochemical Characterization of soil bacteria

2.2.1. Gram Staining: Gram staining was detected by method described by **(Cappuccino and Sherman, 2002):**

2.2.2. Indole test (Morello, *et al*., 2002): Two ml of peptone water was inoculated with 5ml of bacterial culture and incubated for 48 hours. 0.5ml of Kovac's reagent was added shakes well and examined after 1 minute. A red colour in the reagent layer indicated Indole. In negative case there is no development of red colour

2.2.3. Methyl red (MR) and Voges Proskauer (VP) test (Morello, *et al*., 2002): MRVP broth was prepared and 5ml broth is transfer in culture tubes inoculated with bacterial strains and two test tubes were kept as control. The test tubes were incubated at 28ºc for 48h.Five drops of MR indicator were added to each test tube including the control and observed the change in colour similarly ten drops of VP-I reagent and 2-3 drops of VP-II reagent were added to other incubated test tubes and control also. Observed the test tubes for colour change and compared with control.

2.2.4. Citrate utilization test (Ruchhoft, *et al*., 1931): The isolates were carried out by inoculating the test organism in test tube containing Simon's citrate medium and this was inoculated for 24 hours to 72 hours. The development of deep blue colour after incubation indicates a positive result.

2.2.5. **Catalase activity (Taylor and Achanzar, 1972):** The isolates were streaked on yeast extract mannitol agar slant and incubated for 3 days at 28°C. The formation of effervescence upon addition of 1ml of 1% hydrogen peroxide was positive to catalase activity.

2.2.6. Oxidase production (Gaby and Hadley, 1957): The isolates were streaks on yeast extract mannitol agar plates and incubated for 3 days at 28°C. After incubation, a loop full of isolates was placed over oxidase disc (N, N – Tetra methyl-Para-phenyl diamine dihydro chloride). Development of blue (or) purple colouration was positive to oxidase production.

2.2.7. Urease activity (Mac Faddin, 2000): The isolates were streaked on Christensen's urea agar slants and incubated for 3 days at 28°C Observed the slant for a colour change at 6 hours, 24 hours, and every day for up to 6 days. Urease production is indicated by a bright pink colour on the slant that may extend into the butt.

2.2.8. Antibiotic susceptibility test (Baure, *et al*., 1996): The strains were cultured on slants of the solidified Nutrient agar medium and suspended directly in sterile saline without any change in the antibiotic resistance pattern. Antibiotic disks (3disks/plate) were applied on the plates with a dispenser and after 3 days' incubation at 37^{0}C. The antibiotic disks used were the following Chloromphencol^{-10}, Chloromphencol^{-25}, and Chloromphenicol^{-50}.

2.3. IAA (Indole-3-acetic acid) production: (Ahmad *et al*., 2005). IAA (Indole-3-acetic acid) was detected by method described by Ahmad 2005.

2.4. Phosphate solubilization test (Chen *et al*., 2006): Phosphate solubilization ability of isolates was detected by spotting them on Pikovskaya's agar plates. The plates were then incubated at 28±1°C for 3 days and then observed for the appearance of clearing zones around the colonies (due to solubilization of inorganic phosphate by producing organic acid by bacteria).

2.5. Siderophores production: The *pseudomonas* isolates were inoculated in King's B broth and incubated for 15 days. After 15 days' incubation period the culture was centrifuged at 10,000rpm to 15min cell free supernatant were used for the following tests.

2.5.1. Siderophore assay: Siderophore assay was carried out based on the CAS shuttle assay of (Payne 1994). The siderophore content in the aliquot was calculated by using following formula % of siderophore units= $\frac{(Ar-As)\times 100}{Ar}$ Where, Ar= absorbance of reference at 630 nm (CAS reagent) As= absorbance of sample at 630 nm

2.5.2. Ferric chloride test: To 0.5ml of culture filtrate 0.5ml of 2% aqueous ferric chloride solution was added. Appearance of reddish brown /orange colour was positive indication of siderophore production.

2.5.3. Arnow's test (Arnow, 1937): To 1ml of culture filtrate 1ml of 0.5N hydrochloric acid, 1ml of nitrite molybdate reagent and 1ml of 1N sodium hydroxide were added. The formation of red coloured solution was considered as indication of the presence of catechol type of siderophore.

2.5.4. Tetrazolium test (Snow, 1954): To a pinch of tetrazolium salt were added to 2drops of 2N sodium hydroxide and 0.1ml of the culture filtrate. Appearance of a deep red color was indication of hydroxamate type of siderophore.

2.6. Molecular Identification of Efficient Rhizosphere Isolate Using 16S rRNA Gene Sequencing: Molecular characterization of the efficient PGPR isolate was done by 16Sr RNA gene sequencing. DNA of the isolated was extracted by modified method of Sam brook and Russel. The gene was amplified using universal bacterial 16S rRNA gene primers under following conditions; Initial denaturation at 95ºC for 5mins, 30 cycles of denaturation at 95ºC for 30 secs, annealing at 52ºC for 30 secs, extension at 72ºC for 2mins and final extension for 10mins in Eppendorf thermos cycler. The amplified products were purified by electrophoresis in 1.2% agarose and extracted by QIA quick gel elution kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purified PCR products were sequenced.

2.7. Isolation of fungal pathogen *Fusarium* spp: *Fusarium* species were isolated based on its colony and morphological characteristics (Singh *et al*., 1991). Isolated colonies were sub cultured onto potato dextrose agar (PDA) slants and stored at 4^0C. Six day old cultures were used throughout the study.

2.8. Antagonistic activity of *Pseudomonas* sp Bio control assay: Sterile Potato Dextrose Agar was prepared and solidified in petridishes. The well size 6mm diameter was made aseptically in the agar plate. Forty µ1 of partially purified siderophore was added to the well and allowed to diffuse radially for 1 hour at low temperature. The inoculums size of 6×10^3 spores of plant pathogenic fungi was swabbed over the surface of the PDA plates and incubated at room temperature for 48 hours. After incubation, the zone of inhibition of the mycelia growth was measure.

2.9. Pot culture: Seeds of *Sesamum indicum* were soaked in water overnight and surface sterilized with 0.1% mercury chloride for 5min and washed several time with sterilized distilled water. Garden soil and sand (2:1) w/w was stream sterilized for 3 days and then distributed in each parts and then seeds were sown. After germination the seedling were thinned out two, three. The pots were arranged over a slap in the green house. The plants were irrigated with nitrogen free sterile a tap water on alternate days, Plants were harvested at 45 DAI (Day after Inoculation) and separated into leaves, shoot and roots**.** The plant length (cm) was calculated in bio inoculants treated plants and non-inoculants plants. Three plants were taken each pot to measure the mean value for all the treated and control plants.

2.10. Estimation of photosynthetic pigments (mg/g): Chlorophyll a, b and carotenoids contents were extracted from respective dose of leaves and estimated according to the method of (Arnon 1949) and the carotenoids content was determined according to the method of (Krick and Allen 1965). Chlorophyll content was calculated using the formula of Arnon (1949).

2.11. Soil microbial population (cfu/g.soil): Microbial population in the soil samples was determined by the dilution plate's techniques. The results were expressed as colony forming units per gram soil. Dry weight basis, Nutrient Agar (NA), Rose Bengal agar medium (RBA), Potato dextrose agar medium (PDA).Each culture medium was also used in the liquid form without added for dilution preparation. In addition, soils from sites of *in vitro* study area.

2.12. Statistical analysis

The data was subjected to statistical analysis by using Costat package for one-way ANOVA and Student Newman Kauls test.

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3. RESULTS

Rhizosphere soils were collected from Salem district, Tamil Nadu. The soil sample was analyzed for pH, Electrical conductivity, and NPK analysis (Table 1). Bacterial isolates was isolated by serial dilution method. The isolates were biochemical test (Table 2). and followed by genomic DNA identification. (Fig 1) These isolates were conformed with *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Bacillus cereus* they were named as from PSA01, PSA02, BAC03. (Fig 2 ABC). Phosphate solubilizing activity was shown by the isolates PSA02 with a zone of clearance 0.5mm (Fig 2 F). In PS01and PS02 IAA test was positive (Fig 3 E). The siderophores production was assayed qualitatively and quantitatively through Chrome Azural S and the results showed positive for the strains PSA01 and PSA02 that grown in succinate medium (Fig 2 D). In pot culture studies *Sesamum indicum. L* showed significantly increase in the root length, shoot length, fresh weight, dry weight and total chlorophyll and carotenoids significantly increased in treated plant. (Fig 4,5.). The antagonist activity of the siderophore producing *Pseudomonas* spp against fungal pathogen *Fusarium oxysporum.* (Fig 2 F) This result showed that *Pseudomonas* spp is a good producer of siderophore which can be usefully exploited as a biocontrol agent and also as plant growth promoter and increasing the Oil yielding crops.

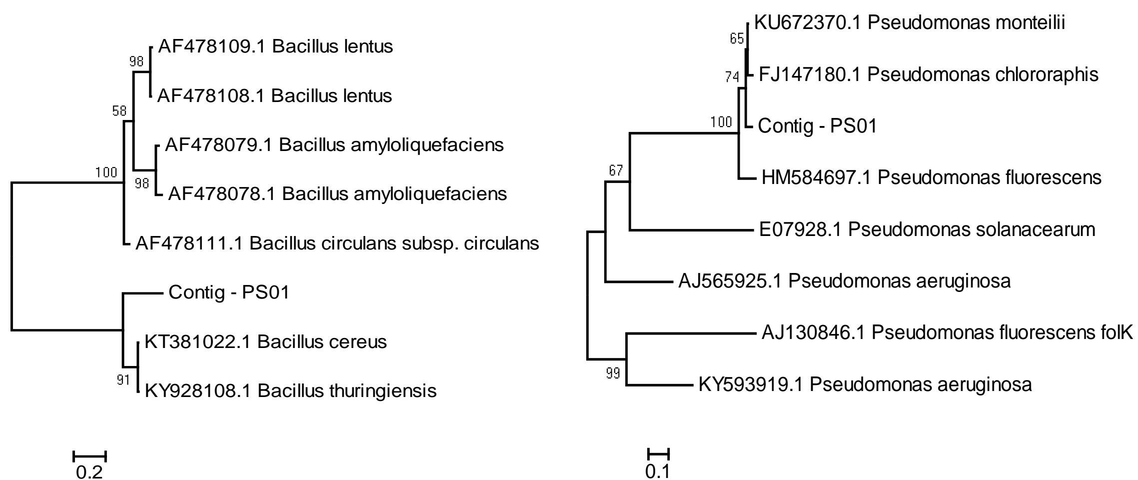

FIG.1

Phylogenetic tree of 16S r RNA gene sequences showing the realationships among the isolates isolated from the soils of plant rhizosphere region Salem district, Tamil Nadu. The data of type strains of related species were from GenBank database (the accession numbers are given in parentheses). Bacillus *cereus* and were submitted GenBank under Accession number MH128361. *Pseudomonas aeruginosa* strain Accession number MH128359.

TABLE 1: BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATION

S.No	Test	PSA01	PSA02	BAC03
1.	Gram staining	_	_	_
2.	Indole production	+	+	_
3.	Methyl Red	+	+	+
4.	Voges-proskaur	_	_	_
5.	Citrate utilization	+	+	+
	Extra-cellular enzymes			
6.	Catalase activity	+	+	+
7.	Oxidase production	+	+	+
8.	Urea's activity	+	+	+

(+)- Positive, (-) Negative

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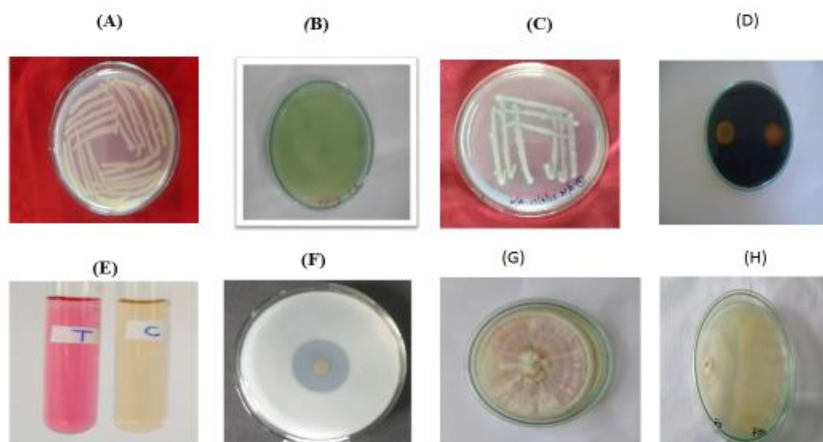

Fig 2: (A) ***Pseudomonas aeruginosa*****. (B)*****Pseudomonas fluorescence*****. (C)*****Bacillus cereus*****(D) Siderophore test. (E) IAA Test.(F) Phosphate solubilization test (G)** ***Fusarium*** **spp (H) Antagonistic activity**

Pot culture studies

After 45 days of inoculation (DAI), compared to isolates BAC03, PSA02&PSA01 significantly enhanced the plant shoot length, root length and leaf length. Inoculation of PSA02 increased *Sesasmum indicum.L* root length (11.5cm) when compared to that of control (10cm) & BAC03 (10.5). PSA02 increased shoot length (26cm), similarly PSA01 also increased shoot length (24.4cm) when compared to that of control (19.3cm) & BAC03 (19.9). PSA02 increased leaf length (4.1cm), similarly PSA01 also increased leaf length (4cm) when compared to that of control (3.1cm) & BAC03 (3.5). Fresh weight of *Sesasmum indicum.L* enhanced PSA02 (12.1g) PSA01 (10.2g) while comparing with control (1.8g) BAC03 (5.8g). Similarly, dry weight also remarkably increased when compared with control (0.5g) & BAC03 (2.4g) PSA02 dry weight (6.4g) PSA01 dry weight (5.2g).

Fig 3: pot culture studies on ***Sesamum indicum*****.L**

TABLE 2: GROWTH PARAMETER OF *(SESASMUM INDICUM .L)* 45 DAI(DAY AFTER INOCULATION)

Treatment	Leaf length (cm)Mean±SD	Shoot length (cm)Mean±SD	Root length (cm)Mean±SD	Fresh weight(g)	Dry weight(g)
Control	0.3±0.1	0.3±0.1	1.5±0.5	0.9±0.75	0.3±0.41
PSA02	**4±1.80**	**17.2±1.71**	**3.6±1.40**	**1.7±1.90**	**0.7±0.43**
PSA01	2±1	4±1.80	1.3±0.44	1.1±0.90	0.07±0.33
BAC03	3.2±0.57	2±0.70	1.5±0.2	1.3±0.02	0.07±0.01

TABLE 3: ESTIMATION OF TOTAL CHLOROPHYLL & CAROTENOIDS FOR (*SESAMUM INDICUM.L*)

Treatment	Total Chlorophyll mg g-^{1}	Carotenoids mg g-^{1}
Control	0.056±0.056	0.26±0.04
PSAO2	**3.981±0.012**	**0.91±0.19**
PSAO1	3.678±0.229	0.04±0.02
BACO3	3.674±0.241	0.52±0.05

TABLE 4: IMPACT OF AFTER INOCULATED SOIL COUNTING OF MICROBIAL POPULATION AT 45 DA

S.no	Treatments	Total bacterial population in soil (cfu/g soil)
1	**Control**	1.3 X10^{7}
2	**PSA01**	4.1 X10^{7}
3	**PSA02**	6.5 X10^{7}
4	**BCA03**	3.2 X10^{7}

Cfu – Colony forming unit

4. DISCUSSION

Pseudomonas gram negative, gram staining test it was confirmed that isolated bacteria belonged to genus *Pseudomonas*. In Gram's staining, the morphology of isolated *Pseudomonas* strains showed Gram-negative, pink colored, medium rod shaped appearance. These findings agreed with the findings reported by earlier researchers (Tripathi *et al*., 2011). In this study, the qualitative estimation of siderophores by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* isolates showed that they were powerful producer of siderophores under limited iron on King's B medium. The production of siderophores by *Pseudomonas fluorescens and Pseudomonas aeruginosa* isolates indicated that these bacteria isolates can be used biocontrol against soil borne phytopathogens. Similarly, reported that *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* as siderophore producer on King's B medium. *Pseudomonas fluorescens* was able to give higher yields of siderophores under iron stress conditions (Sayyed *et al*., 2005). Application of growth parameter of *Pseudomonas fluorescence's* and *Pseudomonas aeruginosa* isolates on *Sesamum indicum* seed was brought significant increases on growth and yield of crop.

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