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ORIGINAL RESEARCH ARTICLE

Studies on the Phytohormone Producing Potential of Agriculturally Beneficial Microbial (ABM) Isolates from Different Rhizosphere Soils of Sunflower in Tamil Nadu

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ABSTRACT

In the present study, agriculturally beneficial microorganisms *viz*., *Azospirillum lipoferum*, *Bacillus megaterium* and *Pseudomonas fluorescens* were isolated from the rhizosphere of sunflower in 30 different locations of Tamilnadu, India. The thirty isolates were further tested for the production of Indole acetic acid (IAA), Gibberellic acid (GA_3) and siderophores. *Azospirillum lipoferum* SA-17 strain (89.9 µg 25 ml^{-1} of broth and 8.62 µg 25 ml^{-1} of broth) produced the maximum amount of IAA and GA_3 in Nitrogen free malate broth, which was followed by *Pseudomonas fluorescens* SP-10 (70.8 µg 25 ml^{-1} of broth and 7.40 µg 25 ml^{-1} of broth) and *Bacillus megaterium* SB-17 strain (63.0 µg 25 ml^{-1} of broth). Among the different isolates tested, *Pseudomonas fluorescens* produced the maximum amount of Siderophore (Catechol 8.26 µg ml^{-1} and Salicylate 8.96 µg ml^{-1}) which was followed by Bacillus megaterium (Catechol 7.80 µg ml^{-1} and Salicylate 7.86 µg ml^{-1}) and Azospirillum lipoferum (Catechol 4.80 µg ml^{-1} and Salicylate 8.40 µg ml^{-1}).

Key words: Sunflower, Indole acetic acid, Gibberellic acid, Siderophore and Agriculturally beneficial microorganisms.

1. INTRODUCTION

Sunflower is an important oilseed crop for its premier oil and manifold uses of both industrial and pharmaceutical importance. Its cultivation has gained momentum due to its special features like short duration, photoperiod insensitivity, drought tolerance, adaptability to wide range of soil and climatic situations, lower seed rate, high content of quality cooking oil and high seed multiplication ratio. The exponential growth of area under sunflower cultivation is an unparalleled example for any crop and this stands testimony for its suitability to fit to different cropping systems and patterns in the country [1].

Agriculturally beneficial microorganisms have been identified in influencing the growth and yield of many plants. Plant growth promoting rhizobacteria increases the plant growth directly or indirectly [2, 3]. Direct mechanisms by PGPR includes, nitrogen fixation, production of Phytohormones such as indole acetic acid, Gibberellic acid, Siderophore production, lowering of ethylene concentration and solubilization of phosphorous, whereas, antibiotic production, depletion of iron from rhizosphere, synthesis of antifungal metabolites, synthesis of antifungal cell wall lysis enzymes, competition for the sites on the roots and induced systemic resistance, are included in the indirect mechanisms of plant growth promotion by PGPR.

Microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils [4]. Plant morphogenic effects may also be a result of different ratios of plant hormones produced by roots as well as by rhizosphere bacteria [5]. IAA (indole-3-acetic acid) is the member of the group of Phytohormones and is generally considered the most important native auxin [6]. It functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation [7].

Diverse bacterial species possess the ability to produce the auxin Phytohormone IAA.

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Gibberellins are known to be a component of light signaling [8] phytochromes and GA_3 act in coordination to regulate multiple aspects of Arabidopsis development such as flowering and hypocotyls elongation [9, 10]. Production of plant hormones by *Azospirillum* [11] as well as the concomitant changes in plant growth and development was observed. Siderophores are low molecular weight bio-molecules secreted by micro-organisms in response to iron starvation for acquisition of iron from insoluble forms by mineralization and sequestration [12]. Although, some siderophores are known to chelate other ions, their specificity and avidity for iron is the most consistent feature [13]. Siderophore producing microorganisms like *Pseudomonas* sp. [14], *Bacillus megaterium* [15] plays the vital role in stimulating plant growth and controlling several plant diseases [16]. They function as a biocontrol agent by depriving the pathogen from iron nutrition, thus resulting in increased yield of crop [17]. The present work focuses on the Indole acetic acid, Gibberellic acid and Siderophore producing potential of agriculturally beneficial microbial strains (*Azospirillum lipoferum*, *Pseudomonas fluorescens and Bacillus megaterium*).

2. MATERIALS AND METHODS

Survey for the collection of rhizosphere soil sample of Sunflower

Survey was conducted at three different locations of Tamil Nadu *viz*., Cuddalore, Villupuram and Thiruvannamalai districts. Thirty rhizosphere soil samples, ten from each district were carefully collected, transported to the laboratory and collected rhizosphere soil samples were stored in refrigerator at 4ºC for further studies.

Biochemical characterization of the test isolates

All the 30 isolates of *Azospirillum lipoferum, Pseudomonas fluorescens and Bacillus megaterium* were biochemically characterized for Gram reaction, Motility, Nitrite reduction, Acid production, Voges-Proskauer test, Citrate utilization, Catalase test, oxidase test, starch hydrolysis, and gelatin liquefaction as per the standard methods [18].

Production of Phytohormones by agriculturally beneficial microbial isolates

The *in vitro* production of Phytohormones such as indole acetic acid (IAA), gibberellic acid (GA_3) and Siderophore by agriculturally beneficial microbial isolates were estimated.

Estimation of indole acetic acid (IAA)

A quantity of 100 ml of nitrogen free malate broth (without bromothymol blue indicator) for *Azospirillum*, nutrient broth for *Bacillus*, and King's B broth for *Pseudomonas* isolates were prepared and sterilized. Freshly prepared, filter sterilized solution of L-tryptophan was added to each flask to a final concentration of 100 mg L^{-1}. One ml of culture broth of agriculturally beneficial microbial isolates were inoculated to each flask and incubated at 37ºC in dark for seven days. After incubation, the cultures were centrifuged at 6,000 g for 5 min to remove the bacteria cells. The supernatant was brought to pH 2.8 with 1 N HCl. Fifteen ml of the acidified supernatant was taken in 100 ml conical flask and to it equal volume of diethyl ether was added and incubated in dark for 4 hrs. IAA extraction was done at 4ºC in a separating funnel using diethyl ether. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, two ml of methanol was added, pooled and the IAA present in the methanol extract was determined using the method of Gorden and Paleg [19]. To 0.5 ml of the methanol extract, 1.5 ml of distilled water and four ml of Salper's reagent (1.0 ml of 0.5 M $FeCl_3$ in 50 ml of 35 per cent per chloric acid) were added and incubated in dark for one hour. The intensity of pink colour developed was read at 535 nm in a spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity in the culture filtrate was determined and expressed as µg 25 ml^{-1} of culture medium.

Estimation of gibberellic acid (GA_3)

The gibberellic acid production by agriculturally beneficial microorganisms was determined by following the method of Borrow *et al.* [20]. A quantity of 100 ml of nitrogen free malate broth (without bromothymol blue indicator) nutrient broth, and King's B broth were prepared for *Azospirillum*, *Bacillus* and *Pseudomonas* isolates respectively and sterilized.

One ml broth of 30 isolates of *Azospirillum*, *Bacillus* and *Pseudomonas* were added separately in the respective medium and incubated at 37ºC for seven days. After seven days of incubation, the culture was centrifuged at 8000 g for 10 min to remove the bacterial cells. Fifteen ml of the culture was pipetted out separately into the test tubes and two ml of zinc acetate solution was added. After two min, two ml of potassium ferrocyanide solution was added and centrifuged at 8,000 g for 10 min.

Five ml of supernatant was added to five ml of 30 per cent hydrochloric acid and the mixture was incubated at 27ºC for 75 min. The blank was prepared with five per cent hydrochloric acid. Absorbance was measured at 254 nm in a UV-VIS spectrophotometer. From the standard graph prepared by using gibberellic acid solutions of known quantities, the amount of GA produced by the culture was calculated and expressed as µg 25 ml^{-1} broth.

Estimation of siderophore production

Siderophore production by the agriculturally beneficial microorganisms was estimated by the method described by Reeves *et al.* [21]. Nitrogen free malate broth, nutrient broth, and King's B broth were prepared for *Azospirillum*, *Bacillus* and *Pseudomonas* isolates respectively and dispensed in 100 ml quantities in 250 ml Erlenmeyer flasks and sterilized. One ml of standard inoculum of bacterial isolates was added into each flask and incubated at 37ºC for 7 days. After seven days of incubation, the culture broth was centrifuged at 10,000 g for 20 min. The supernatant was used for estimation of siderophores. Twenty ml of culture supernatant was taken and the pH was adjusted to 2.0 with dilute HCl. To 20 ml of supernatant, 20 ml of ethyl acetate was added and extraction was done twice. Hathway reagent (one ml of 0.1 M ferric chloride and 1 ml of 0.1 N HCl was added to 100 ml of distilled water and to this 1 ml of 0.1 M potassium ferricyanide was added) was prepared. Five ml of the assay solution was added with five ml of Hathway reagent and absorbance was determined at 560 nm with sodium salicylate as standard for the estimation of salicylate type of siderophore. To measure the catechol type siderophore, five ml of the assay solution was added with five ml Hathway reagent and absorbance was determined at 700 nm with 2, 3 dihydroxy benzoic acid (DHBA) as a standard. One µ mole of 2, 3-DHBA gave an absorbance of 0.75. From the absorbance value of the sample, the quantum of siderophore produced was calculated and expressed as µg ml^{-1} of culture filtrate.

3. RESULTS AND DISCUSSION

A total of 30 isolates of *Azospirillum lipoferum*, *Pseudomonas fluorescens* and *Bacillus megaterium*, were isolated from rhizospheric soils and tentatively identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergey's manual of Determinative Bacteriology. These bacterial isolates were screened for their ability to produce plant growth regulators IAA, GA_3 and Siderophore production.

All the thirty isolates of Azospirillum lipoferum produced IAA and GA3 and the quantity ranged from 2.20 to 89.90 µg 25 ml^{-1} broth for IAA and 2.74 to 8.62 ml^{-1} 25 ml^{-1} broth for GA_3. The isolate SA-17 produced the maximum amount of IAA 89.90 25 ml^{-1} broth and 8.62 25 ml^{-1} broth for GA_3 of nitrogen free malate broth followed by SA-23 which produced 76.22 µg of IAA 25 ml^{-1} broth and 7.26 25 ml^{-1} broth for GA_3.

All the *Azospirillum* isolates produced both catechol and salicylate type of siderophores. The catechol type of siderophore produced by *Azospirillum lipoferum* isolates ranged from 1.54 to 4.80 µg ml^{-1} of culture broth and salicylate type ranged from 2.30 to 8.40 µg ml^{-1}.The isolate SA-17 produced the highest quantity of 4.80 and 8.40 µg ml^{-1} of catechol and salicylate type of siderophores respectively followed by other isolates (**Table 1**). The potential of *Azospirillum* strains to produce Indole-3-acetic acid and gibberellic acid under *in vitro* conditions was reported by Bashan *et al.* [22] and Pedraza *et al.* [23]. Among the thirty *Bacillus* isolates, the isolate SB-17 produced the maximum amount of 63.00 µg IAA 25 ml^{-1} and 6.80 µg 25 ml^{-1} broth for GA_3 followed by SB-18 isolate which produced 56.0 µg IAA 25 ml^{-1} and 6.20 µg 25 ml^{-1} broth for GA_3. Among the thirty *Bacillus* isolates, the isolate SB-17 recorded the maximum production of both catechol and salicylate type of siderophores (7.80 and 7.86 µg ml^{-1}) followed by other isolates (**Table 2**).

All the thirty isolates of *Pseudomonas fluorescens* produced IAA and $GA_3$and the quantity ranged from 15.60 to 70.8 µg 25 ml^{-1} broth for IAA and 2.80 to 7.40 µg 25 ml^{-1} broth for GA_3. The isolate SP-10 produced the maximum amount of 70.8 IAA 25 ml^{-1} and 7.40 µg 25ml $^{-1}$ broth for GA_3 followed by SP-17 which produced 68.23 µg of IAA 25 ml^{-1} and 7.0 µg 25 ml^{-1} broth for GA_3.The siderophore production by *Pseudomonas fluorescens* SP-10 recorded the maximum siderophore production of 8.26 and 8.86 µg of

catechol and salicylate type ml^{-1}. The minimum amount of siderophore production was recorded with the isolate SP-27 of about 3.12 and 8.96 µg ml^{-1} (**Table 3**). Our test isolates showed a similar high level of IAA and GA_3production to those recorded by other researchers [24, 25]. The IAA and GA_3 producing potential of *Azospirillum* isolates was more than that of *Pseudomonas* and *Bacillus* isolates. It is also to note that *Pseudomonas* isolates were better than *Bacillus* for IAA and GA3 production. The production of IAA and $GA_3$by *Bacillus* and *Pseudomonas* in the rhizosphere of wheat and vegetables and their plant growth promoting role was well explained by Verma *et al*. [26]. Tank and Saraf [27] in various plant rhizosphere.Indole-3-aceticacid is a phytohormone which is known to be involved in root initiation, cell division and cell enlargement [24, 25]. All the isolates of *Azospirillum*, *Bacillus* and *Pseudomonas* produced appreciable quantities of siderophore. Among the plant growth promoting bacteria, *P. fluorescens* SP-10 secreted highest amount of both catechol and salicylate type of siderophores followed by *Bacillus* and *Azospirillum* isolates.

Production of siderophore by plant growth promoting bacteria and its role in Fe mobilization was reported by several workers [28-33]. All the plant growth promoting bacterial isolates tested in the present study produced more amount of salicylate type than catechol type of siderophore. The potential of *Pseudomonas* to produce siderophore was more than that of *Bacillus* and *Azospirillum.*

Table 1: Indole acetic acid (IAA), Gibberellic acid (GA_3) and Siderophore Producing Potential of *Azospirillum* isolates obtained from the rhizosphere of sunflower

Isolate number	IAA (µg 25 ml^{-1} of broth)	GA_3 (µg 25 ml^{-1} of broth)	Siderophore content (µg ml^{-1})	
			Catechol Type	Salicylate Type
SA-1	72.3	6.80	3.38	6.72
SA-2	46.7	4.74	3.84	6.82
SA-3	22.1	2.99	2.28	4.84
SA-4	54.0	5.62	3.68	6.20
SA-5	32.6	3.14	1.98	2.80
SA-6	29.8	2.74	3.72	6.96
SA-7	33.8	3.97	1.58	3.26
SA-8	44.9	4.12	2.70	4.90
SA-9	26.3	2.92	2.00	4.21
SA-10	21.2	3.02	3.41	6.23
SA-11	30.0	3.16	1.54	2.30
SA-12	32.0	3.76	2.89	5.00
SA-13	22.1	3.00	3.50	6.81
SA-14	59.0	6.21	3.10	6.00
SA-15	29.8	3.04	2.92	4.82
SA-16	74.0	7.02	4.52	7.82
SA-17	89.9	8.62	4.80	8.40
SA-18	69.8	6.90	2.72	5.12
SA-19	49.0	5.20	2.00	4.88
SA-20	46.2	5.00	4.48	7.80
SA-21	64.0	6.42	2.80	4.50
SA-22	68.4	6.98	4.60	7.28
SA-23	76.0	7.26	2.90	4.80
SA-24	52.0	5.22	1.59	3.22
SA-25	27.0	2.16	2.85	4.68
SA-26	37.0	3.72	3.97	6.11
SA-27	32.0	3.19	3.14	5.40
SA-28	53.8	5.12	1.26	2.80
SA-29	42.8	3.98	3.80	6.18
SA-30	69.7	6.93	3.98	6.90
SE_D	3.50	0.31	0.17	0.29
CD(P=0.05)	7.06	0.65	0.36	0.60

Table 2: Indole acetic acid (IAA), Gibberellic acid (GA_3) and Siderophore producing potential of *Bacillus* isolates obtained from the rhizosphere of sunflower

Isolate number	IAA (µg 25 ml^{-1} of broth)	GA_3 (µg 25 ml^{-1} of broth)	Siderophore content (µg ml^{-1})	
			Catechol Type	Salicylate Type
SB-1	54.6	5.8	7.12	7.36
SB-2	39.2	4.0	4.19	4.99
SB-3	41.0	4.3	5.80	6.23
SB-4	43.2	4.5	6.21	6.89
SB-5	31.0	2.9	3.84	4.18
SB-6	40.0	3.8	5.83	6.20
SB-7	33.8	3.2	5.13	6.12
SB-8	46.0	4.4	7.36	7.20
SB-9	29.8	2.8	3.16	4.00
SB-10	27.8	2.6	4.68	5.14
SB-11	32.3	3.4	5.78	6.23
SB-12	49.8	5.2	3.24	4.60

SB-13	40.0	3.9	4.12	5.82
SB-14	52.0	5.3	4.00	5.32
SB-15	12.0	1.4	2.98	3.54
SB-16	48.0	4.6	3.37	4.20
SB-17	63.0	6.8	7.80	7.86
SB-18	60.6	6.2	7.60	7.40
SB-19	51.8	5.4	5.78	6.27
SB-20	48.7	4.9	6.90	7.00
SB-21	39.8	3.8	3.50	3.87
SB-22	44.7	4.7	6.85	7.24
SB-23	48.2	4.3	6.14	6.89
SB-24	32.8	3.8	4.82	5.14
SB-25	34.7	3.9	4.19	5.02
SB-26	36.4	4.2	6.72	7.00
SB-27	39.8	4.4	5.28	5.98
SB-28	52.8	5.3	6.20	6.82
SB-29	32.6	3.2	4.84	5.25
SB-30	54.0	5.5	3.96	4.23
SE_D	1.98	0.20	0.26	0.22
CD(P=0.05)	4.02	0.42	0.54	0.46

Table 3: Indole acetic acid (IAA), Gibberellic acid (GA_3) and Siderophore producing potential of *Pseudomonas* isolates obtained from the rhizosphere of sunflower

Isolate number	IAA (µg 25 ml^{-1} of broth)	GA_3 (µg 25 ml^{-1} of broth)	Siderophore content (µg ml^{-1})	
			Catechol Type	Salicylate Type
SP-1	59.6	6.3	5.89	6.81
SP-2	47.2	5.2	6.10	7.02
SP-3	30.0	4.3	3.96	4.30
SP-4	17.6	3.8	4.75	5.20
SP-5	68.2	7.0	6.82	7.24
SP-6	32.3	5.0	6.20	7.21
SP-7	52.8	6.0	3.24	4.75
SP-8	40.2	5.3	7.80	8.24
SP-9	15.8	4.2	5.23	6.75
SP-10	70.8	7.4	8.26	8.96
SP-11	25.8	2.7	6.27	7.20
SP-12	52.0	5.8	7.31	8.24
SP-13	46.8	4.9	3.34	4.86
SP-14	38.7	4.3	7.63	8.25
SP-15	60.1	6.3	8.00	7.96
SP-16	29.4	3.9	4.40	5.80
SP-17	68.2	7.0	7.43	8.24
SP-18	50.4	6.2	4.00	4.89
SP-19	38.7	4.2	7.30	8.00
SP-20	59.8	5.8	3.75	4.82
SP-21	52.8	5.3	6.08	6.86
SP-22	32.3	4.7	7.52	8.14
SP-23	40.2	4.0	6.25	7.72
SP-24	29.0	2.9	4.21	5.00
SP-25	17.6	3.2	6.93	7.20
SP-26	38.7	4.2	5.58	6.38
SP-27	46.4	5.9	3.12	4.22
SP-28	22.4	3.8	6.25	7.32
SP-29	15.6	2.8	5.52	6.75
SP-30	62.0	6.2	6.34	7.23
SE_D	3.02	0.23	0.28	0.25
CD(P=0.05)	6.07	0.48	0.57	0.51

4. CONCLUSION

The findings of the present investigation highlighted that IAA, GA_3 and siderophore producing potential of agriculturally beneficial Microbial inoculants from rhizospheric soils could be easily isolated and may be exploited after strain improvement for research. However, further studies using ABM strains of these isolates are needed to explore the exact contribution of IAA, GA_3 and siderophore production in the promotion of plant growth as well as the contribution of other agriculturally beneficial Microbial traits.

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