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Research Article

In vitro Screening of Pink Pigmented Facultative Methylotrophs Based on their Functional Characteristics

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ABSTRACT

Investigations were carried out to study the plant growth promotional ability of native pink pigmented facultative methylotrophs (PPFMs) of major DSR growing areas of Hyderabad-Karnataka. Selected isolates were screened for beneficial characters like production of phytohormones (IAA), In Vitro Nitrogen Fixation, phosphate solubilisation and HCN production. All the isolates tested were found to produce the plant hormone indole acetic acid, nitrogen fixation and phosphate solubilisation. Higher indole acetic acid production and in vitro nitrogen fixation was recorded in PPFM-31 (28.23 µg ml^{-1} of culture filtrate and 1.32 mg N/g of malate) respectively) followed by ppfm-16 recorded IAA production and in vitro nitrogen fixation of 26.15 µg ml^{-1} of culture filtrate and 1.18 mg N/g of malate respectively while reference strain (Methylobacterium extorquens) recorded IAA production and in vitro nitrogen fixation of 26.25 µg ml^{-1} of culture filtrate and found to fix 1.19 mg N/g of malate respectively. PPFM isolates showed wide variation in nitrogen fixation ranging from 0.39 to 1.32 mg N/g of malate. Mineral phosphate solubilisation index of PPFM isolates ranged from 2.10 to 14.95 mm. Among the strains, PPFM-31, reference strain and PPFM-16 showed the solubilization zones of 14.95, 13.00 and 12.30 mm respectively. The present study has identified potential native PPFM strains from major Direct seeded growing districts of Hyderabad- Karnataka for their exploration in improving production and productivity of direct seeded rice.

Key words: *PPFM's, Methylobacterium, IAA, In Vitro Nitrogen Fixation, phosphate solubilisation, HCN production and DSR.*

INTRODUCTION

Bacteria by far the most important a bundant organisms in the soil and they play a key role in nutrient cycling and soil fertility. Various interactions occur between bacteria and plant roots that can beneficial, neutral or harmful. Several rhizo bacterial strains have been found to increase plant growth, called plant growth promoting rhizo bacteria (PGPR).

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In the similar ways suppressive of diseaes provoking microorganisms can occur through microbial antagenses in the rhizosphere. Methylotrophic bacteria is successful example that can achives the two purposes as PGPR with antifungal activity. Many of the microbes living on the phylloplane probably lead a saprophytic lifestyle, feeding on materials leached from the leaf. One such example is *Methylobacterium* sp. a pink pigmented facultative methylotroph (PPFM) which was first identified as covert contaminants from the tissue culture of liverwort, *scapania nemorosa*[3]. This bacterium provides a useful model for the unappreciated kinds of interactions between plants and bacteria that take place routinely on lab and in culture dishes[8]. The genus *Methylo bacterium* is composed of a variety of pink pigmented methylotroph (PPFM) which are capable of growing on C_1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substates such as C_2, C_3 and C_4 compounds. PPFMs are ubiquitous in nature and frequently reported on various plant species, those are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves. These bacteria are commonly found in soils, as well as on the surfaces of leaves, seeds and in the rhizosphere of a wide variety of plants, with highest numbers on actively growing and meristamatic tissue[10] Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins and to alter agronomic traits like branching, seedling vigour, rooting and heat/cold tolerance[27].

Pink pigmented facultative methylotrophs (PPFMs) are ubiquitous in nature and are commonly found in soils, as well as on the surfaces of leaves, seeds and in the rhizosphere of a wide variety of plants, with highest numbers present on actively growing and meristamatic tissues[5] and influence plant growth by production of indole-3-acetic acid (IAA), vitamins[3] and cytokinins[16].

Methylotrophs are those microorganisms which are able to grow utilizing the reduced carbon compounds, like methanol (released during plant metabolism) enhance the plant growth by providing it with nitrogen[18]. Whereas PPFMs colonize rhizosphsere as well as phyllosphere and enhance the plant growth by providing plant growth hormones like auxins, siderospore production and P-solubilization[11]. The association of PPFM species with plants seems to rely on a symbiotic relationship between the bacterium and host plants. The most common niche for synergism between *Methylobacterium* and plant is the phyllosphere, where they utilize methanol evolved from leaves as the sole source of carbon and energy[37], and in response, Methylobacteria may produce phytohormones such as cytokinin and auxins[25]. In addition, they can fix atmospheric nitrogen, bring about mineral phosphate solubilization[14] regulate the ethylene level in rhizosphere by 1-aminocyclopropane-1-carboxylate deaminase[23] and stimulate the resistance against plant pathogens[24].

Hence, by considering the importance of PPFM as plant growth promoting bacteria, we isolated and identified the native isolates of PPFM from direct seeded rice fields of major DSR growing districts of Hyderabad-Karnataka in order to assess their growth promotional ability through phytohormones production, in vitro nitrogen fixation and phosphate solubilisation so that they can be further be utilized as potential bioinoculants to improve the growth and yield of direct seeded rice.

MATERIALS AND METHODS

Functional characteristics of pink pigmented facultative methylotroph isolates

Indole acetic acid production

All the isolates were examined for IAA production qualitatively. The IAA production by PPFM isolates was determined following the method of Ivanova *et al*.[11]. One hundred ml quantities of AMS liquid medium was dispensed into 250 ml Erlenmeyer flasks and sterilized at 15 psi for 15 min. Freshly prepared, filter sterilized solution of L-tryptophan was added to a final concentration of 100 mg l^{-1}. One ml of the standard inoculums (10^9 cells ml^{-1}) of PPFM isolates was inoculated to each flask and incubated at room temperature in a shaker. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 7 days of incubation period, 25 ml of the sample was withdrawn and the cells were spun at 5000 g for 15 min in a centrifuge for quantitative estimation of IAA.

Quantitative estimation of IAA production in PPFM isolates by Spectrophotometric method

A known quantity of the sample was taken (0.5ml) in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Sapler's reagent and incubated in dark for 1 hr at 28°C. The intensity of the pink color developed was read in a spectrophotometer at 540 nm. By referring to a standard graph prepared with chemical grade indole-3-acetic acid, the quantity of IAA in the sample was determined and expressed as µg ml^{-1} of culture filtrate[35].

***In vitro* Nitrogen fixation (Microkjeldhal Method)**

To a 250 ml conical flask 100 ml of the N-free malate medium was dispensed and autoclaved. Later one ml of 24 h old culture inoculum was added to each flask. The flasks were incubated at 37 ^oC for seven days.

After 7 days of incubation, 10 ml of homogenized culture was digested with 5 ml of concentrated H_2SO_4 along with 0.2 digestion catalyst mixture K_2SO_4: $CuSO_4$: Selenium (100:10:1). After cooling, volume was made upto 10 ml with distilled water. Later 10 ml of aliquot was transferred to micro kjeldhal distillation unit. The sample was mixed with 20 ml of 40 per cent NaOH and distilled. Ammonia evolved was trapped in 4 per cent boric acid mixed indicator (Bromocresol green 0.066 g and methyl red 0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against 0.05 N H_2SO_4 and total nitrogen was determined and results were expressed as mg N fixed per g of malate[12].

$$\text{Per cent N} = \frac{\text{Titer value} \times 0.014 \times \text{N of } H_2SO_4 \times \text{Volume made}}{\text{Sample used}} \times 100$$

Phosphorous solubilization

Screening of PPFM isolates for MPS activity

All the isolates were subjected to preliminary screening of phosphate solubilization by a plate assay method using Pikovskaya's (PVK) agar medium supplemented with tri calcium phosphate (TCP). The pH of the media was adjusted to 7.00 before autoclaving. The spotted plates were incubated at 28 ± 2^oC and zone of solubilization (mm) were recorded after ten days of incubation[33].

Amount of TCP solubilized by the PPFM isolates

The PPFM isolates showing zone of solubilization on Pikovskaya's agar were further examined for their ability to release Pi from TCP in the broth medium. One ml of overnight culture of each isolates was inoculated to 50 ml Pikovskaya's broth in three replicates. All the inoculated flasks were incubated at 30 ^oC on a temperature controlled shaker. The Pi released in the broth was estimated after 10^{th} day of incubation from

flasks in comparison with the set of uninoculated controls. The broth cultures were centrifuged at 10,000 rpm for 10 min in centrifuge to separate the supernatant from the cell growth and insoluble phosphate. The available P content in the supernatant was estimated by phosphomolybdic blue color method[12].

HCN production

The ability of the antagonistic *Methylobacterium* to produce HCN were assessed as per the method of Wei *et al*.[38]. Whatman filter paper was placed on the lid of the Petri plates and the plates sterilized. CAS medium amended with glycine (4.4 g/litre) was sterilized and poured into the sterile plates. The antagonistis were streaked on the medium. The filter paper padding in each plate was soaked with two ml sterile picric acid solution. The plates were sealed with parafilm in order to contain gaseous metabolite produced by the antagonists and to allow for a chemical reaction with picric acid on the top. After incubation for weeks at 30 ºC, the colour change of filter paper was noted and the HCN production potential of the antagonists was assessed as per the following scoring.

No colour change	: Nil
Brownish colour change	: Weak
Brownish to orange	: Moderate
Completely orange	: Strong

RESULT AND DISCUSSION

Functional characteristics of pink pigmented facultative methylotroph isolates

The functional diversity of all the PPFM isolates were analyzed in terms of IAA, *in vitro* nitrogen fixation and mineral phosphate solubilization (MPS) in order to screen and select the most efficient PPFM isolates for pot culture studies with direct seeded rice.

Indole-acetic acid (IAA) production by PPFM isolates

In the present study all the isolates tested were found to produce the plant hormone indole acetic acid (IAA) in the presence of tryptophan. The isolates showed wide variation in IAA synthesis ranging from 13.80 to 28.23 µg ml^{-1} of culture filtrate.

The significantly higher IAA production of 28.23 µg ml^{-1} of culture filtrate was recorded in PPFM-31, followed by IAA production of 27.50µg ml^{-1} of culture filtrate was recorded in PPFM-4. PPFM-16 recorded IAA production of 26.15 µg ml^{-1} of culture filtrate and was comparable with that of reference strain which recorded IAA production of 26.25 µg ml^{-1} of culture filtrate. The minimum quantity of indole acetic acid production of 13.80 µg ml^{-1} of culture filtrate was recorded in PPFM-1. IAA is known to stimulate division, extension and differentiation of plant cells, enhance root formation by promoting the conversion of parenchyma into xylem and phloem[26].

The IAA production was found to be increased in the presence of IAA precursor *viz.*, tryptophan. Ivanova *et al.*[11], Madhaiyan[22], Senthil kumar[32] and Thangamani and Sundaram[36] have also reported that the presence of tryptophan would increase the IAA production under *in vitro* conditions.

Many epiphytic and soil microorganisms are able to synthesize and secrete auxins, primarily IAA[7,20] due to which they influence the growth of plants. The first report on the production of indole acetic acid in significant amount by four different methylotrophs was reported by Ivanova *et al.*[11]. The production of plant growth regulators like auxins, zeatin and related cytokininns by methylotrophs and their positive influence on seed germination and seedling growth has been reported earlier[30,25]. Jones[13] observed variability among PPFM isolates in promoting IAA ranging from 0.14 to 25.12 µg/ml. Radha *et al.*[28] documented production of IAA by PPFM ranging from 9.04 to 28.15 µg/ml. Similarly, Sheela *et al.*[34] documented production of IAA by PPFM ranging from 22.47 to 29.97 µg/ml.

Nitrogen fixation by PPFM isolates

In the present study, all the isolates were tested for nitrogen fixation. The nitrogen fixation of

PPFM isolates was estimated *in vitro* by Microkjeldhal method[12] in N-free semi-solid malate medium. PPFM isolates showed wide variation in nitrogen fixation ranging from 0.39 to 1.32 mg N/g of malate.

The significantly higher nitrogen fixation of 1.32 mg N/g of malate was observed in PPFM-31 followed by nitrogen fixation of 1.18 mg N/g of malate observed in PPFM-16. Reference strain was found to fix 1.19 mg N/g of malate. The lowest nitrogen fixation of 0.39 mg N/g of malate was recorded in PPFM-3. The results of nitrogen fixation were similar with Radha *et al*.[29] who documented nitrogen fixation ranging from 0.24 to 1.56 mg/g of malate in soybean. Similarly, Araujo *et al*.[1] reported *Methylo bacterium* species, which have the capacity to fix nitrogen, have previously been isolated from citrus, scotch pine and crotalaria, showing the capacity of members of this bacterial genus to colonize the plant habitat.

Members of *Methylobacterium* are associated with nitrogen metabolism in plants by means of bacterial urease[9]. *Methylobacterium* strains are able to establish efficient nitrogen-fixing symbiosis by nodulating legume roots[35]. Raja *et al*., 2006 isolated nodulating and non-nodulating *Methylobacterium* spp. from legumes. Similarly, Madhaiyan *et al*.[24] isolated several nodulating *Methylobacterium* from tropical legumes such as field beans, cowpea, black gram, soybean, *Sesbania* with high nitrogenase activity. These isolates are able to form effective nodules in *Crotolaria juncea*.

Kumar *et al*.[17] reported that the ultimate aim of establishing endophytic interaction between diazotrophic bacteria and non-legumes is to fix N_2 which later transferred the fixed N_2 to the plants. *Azorhizobium caulinodans* and *Methylobacterium* species were capable of N_2-fixing in a free-living condition. It was anticipated that the intercellular colonization of rice might provide a niche for N_2 fixation.

P-solubilization by PPFM isolates

Phosphorus (P), one of the major essential macronutrients for biological growth and development, exists in nature in a variety of organic and inorganic forms[6,31]. Several microorganisms are involved in phosphate solubilization by various mechanisms like, the process of acidification, chelation exchange reactions or by the production of mono-, di- and tricarboxylic acids. *In vitro* studies have shown that phosphate solubilization can be associated with a marked drop in pH, production of phosphatases and organic acids.

Microorganisms are known to effectively recycle inorganic P in rhizosphere. Microbes which have the ability to release Pi from insoluble phosphate present in soil have been advocated to be used in bio-fertilization programme. PPFM have the ability to solubilize inorganic P and PPFM-117 isolated from the Western Ghats was reported for this activity for the first time[14,13]. In the present study, PPFM isolates were screened for their phosphate solubilizing ability. All the isolates solubilized phosphorous ranging from 8.17 to 13.00 per cent. The highest Pi release was achieved by PPFM-31 (13%) followed by PPFM-30 (12.30 %) and PPFM-16 (12.15%) which were higher than that of the reference strain (10.57%). However, PPFM-1, PPFM-3 and PPFM-44 released the lowest amount of Pi (8.17%, 8.33% and 9.57% respectively).

The results showed similarity with the work of Sheela *et al*.[34] who documented Pi release ranging from 7.17 to 9.80 per cent in *Coleus forskohlii*. Similarly Jones[13] documented Pi release ranging from 3.20 to 15.58 per cent with different pH reduction in different PPFM isolates isolated from Grape wines. The result obtained in the present study reflects the possibility of using a single culture to provide both Plant Growth Promoting Substances (PGPS) and Pi in the rhizosphere of DSR.

HCN production by PPFM isolates

All the isolates were tested for HCN production but none of the isolates were able to produce HCN (data not shown).

Table 1: Functional characteristics of pink pigmented facultative methylotroph isolates

Sl. No.	Isolate code	IAA (µg/ml)	*In vitro* nitrogen fixation (mg/g of malate)	Pi -release (%)	HCN production
1	PPFM-1	13.80^{kl}	0.63^{hi}	8.17^{f}	_
2	PPFM-3	22.42^{defg}	0.39^{k}	8.33^{f}	_
3	PPFM-4	27.50^{ab}	0.43^{jk}	9.57^{ef}	_
4	PPFM-5	23.15^{cde}	0.93^{de}	10.23^{e}	_
5	PPFM-8	18.25^{hij}	0.84^{ef}	9.80^{ef}	_
6	PPFM-15	21.40^{defgh}	1.17^{b}	12.13^{abcd}	_
7	PPFM-16	26.15^{ab}	1.18^{ab}	12.15^{abcd}	_
8	PPFM-17	18.65^{hij}	0.48^{jk}	12.10^{abcd}	_
9	PPFM-18	23.47^{cdef}	0.55^{ij}	11.25^{abcde}	_
10	PPFM-19	24.93^{abcd}	0.71^{fgh}	10.50^{de}	_
11	PPFM-22	23.40^{cdef}	0.78^{efg}	10.75^{bcde}	_
12	PPFM-28	20.41^{fghi}	0.85^{def}	9.80^{ef}	_
13	PPFM-29	24.30^{bcde}	0.99^{cd}	9.80^{ef}	_
14	PPFM-30	24.44^{bcde}	0.93^{de}	12.30^{abc}	_
15	PPFM-31	28.23^{a}	1.32^{a}	13.00^{a}	_
16	PPFM-32	17.46^{ij}	1.09^{bc}	10.50^{de}	_
17	PPFM-42	21.24^{efgh}	1.13^{bc}	12.50^{a}	_
18	PPFM-43	16.49^{jk}	1.17^{b}	12.15^{abcd}	_
19	PPFM-44	19.32^{ghij}	0.65^{ghi}	9.57^{ef}	_
20	PPFM-47	20.40^{fghi}	0.75^{fgh}	9.88^{ef}	_
21	Reference strain *(M. extorquens)*	26.25^{abc}	1.19^{ab}	10.57^{cde}	_
S.Em±		1.28	0.05	0.63	
CD (0.05)		3.67	0.14	1.79	

Reference strain PPFM-16 PPFM-31

Plate 3: Pure culture of efficient PPFM isolates

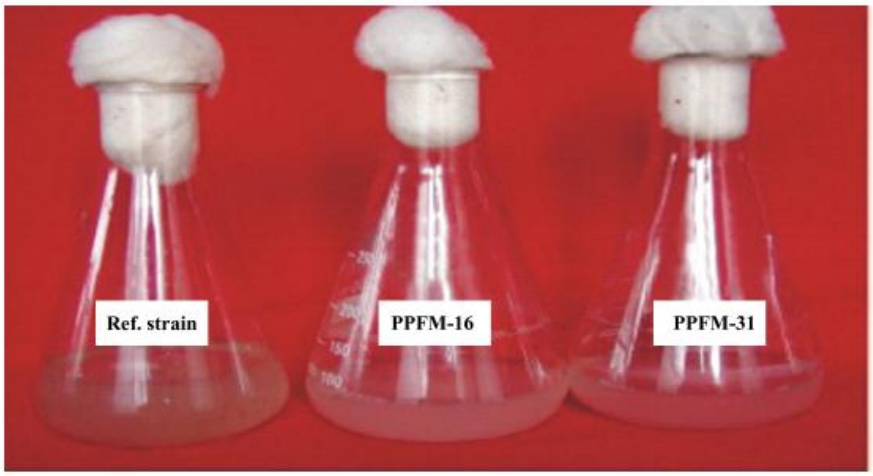

Plate 4: Growth of efficient PPFM isolates on AMS broth

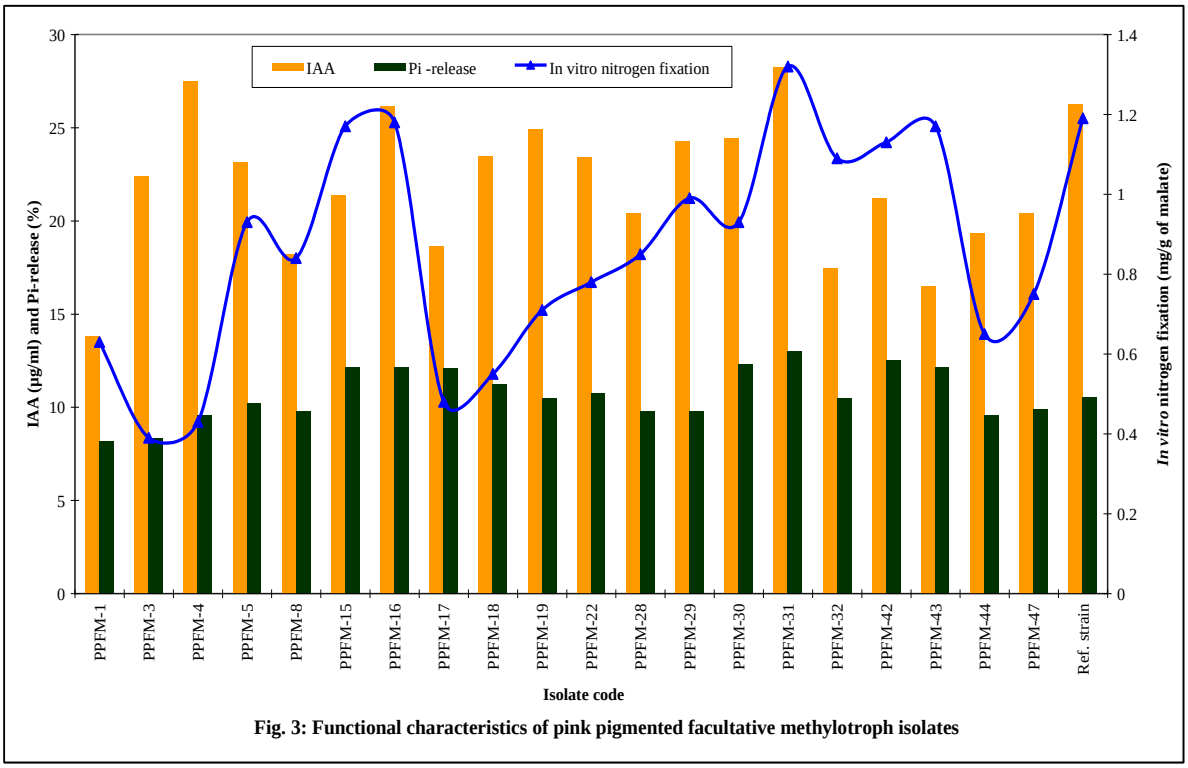

Fig. 3: Functional characteristics of pink pigmented facultative methylotroph isolates

CONCLUSION

The 20 PPFM isolates were screened based on indole acetic acid production, *in vitro* nitrogen fixation and Pi release, along with the reference strains *M. extroquens*. Based on *in vitro* studies out of the twenty isolates, two promising isolates (originally isolated from phyllosphere of DSR) were selected for the pot culture experiments to assess their growth promotional ability and yield parameters of DSR. So these PPFM isolates exhibit characteristics of plant growth promoting microorganisms. PPFM isolates found efficient under *in vitro* conditions *viz*, PPFM-16 and PPFM-31 were selected as best isolates along with reference strain *Methylobacterium extorquens*, we propose further exploration of these identified potential PPFM isolates as bioinoculants in improvement of production and productivity of direct seeded rice in the pot culture study.

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