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Isolation and Characterization of Pink Pigmented Facultative Methylotrophic Bacteria: An *in-Vitro* Evaluation of the Isolates for Plant Growth Promotion on Rice

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ABSTRACT: A total of 50 PPFM's were isolated from rhizosphere soil and phyllosphere of Pink Pigmented Facultative Methylotrophic (PPFM) isolates (IIRRSV22-1 to IIRRSV22-18 and M1) obtained mostly from the phyllosphere of rice plants collected from Telangana were screened for plant growth promoting traits and for tolerance towards abiotic stresses. All isolates were observed to produce indole acetic acid (IAA), while gibberellic acid (GA) production was detected in nine isolates, with the isolate IIRRSV22-5 producing the highest IAA and GA of 10.88 and 10.20 µg/ml of culture filtrate, respectively. Seven and sixteen isolates possessed the ability to solubilise phosphorus and zinc respectively, whereas potassium solubilization was observed only in one isolate (IIRRSV22-8). Isolates IIRRSV22-9 and IIRRSV22-5 recorded the highest solubilization index of 3.12 and 3.50 for phosphorus and zinc respectively. Isolate IIRRSV22-1 possessed the highest siderophore production index (3.42), while isolate IIRRSV22-5 was observed to grow more luxuriantly using ACC as the sole N source compared to other isolates. HCN production was not observed in any of the isolates. All isolates were tolerant to 2% NaCl, while 8 isolates (IIRRSV22-1,3,4,5,11,13,14 and M1) tolerated 3% NaCl concentration. When screened for moisture stress tolerance using PEG 6000 with IIRRSV22-4 isolate recording the highest growth. An *in vitro* germination assay was performed using all the isolates, in which rice seeds (DRR Dhan 46) inoculated with IIRRSV22-5 showed the highest significant increase in seed germination (100%), root length (12.47cm), shoot length (6.49cm) and seedling vigour index (1896) compared to control. The bacterial isolates were scored based on their efficiency to act as PGPR and for their competence to induce a beneficial interaction during *in-vitro* studies with rice. The total score secured by each isolate was computed as the sum of scores across all traits and the isolates with the highest scores were selected as the most promising PPFM. The five isolates with the highest scores were identified as *Methylobacterium aquaticum, Methylobacterium phyllosphaerae, Methylobacterium radiotolerans, Methylobacterium fujisawaense* and *Methylobacterium* sp. Alleviating the negative effects of abiotic stresses like drought, heat and salinity that are responsible for sharp decline in rice yields is a major challenge in the present climate change scenario. A promising eco-friendly strategy for increasing rice yields is the use of microorganisms to reduce abiotic stress on rice. Therefore, the current study has identified PPFM strains that have ability to promote plant growth which can be further used for the betterment of production and productivity of rice.

Keywords: Rice, Phyllosphere, Cytokinins, Pink pigmented facultative methylobacteria (PPFM), Plant growth promotion, Abiotic stress tolerance.

INTRODUCTION

Rice is the chief food for above 65% of the Indian population and contributes to nearly 40% of the entire food grain production. To meet the future rice demand for the growing Indian population, rice production needs to be increased. However, increasing rice production has presently become very challenging due to problems of reduction and degradation of natural resources and climate change. Water which is a key natural renewable resource in rice cultivation is greatly impacted by climate change and scarcity of water is expected to become an ever-increasing problem in India (Palaniswani *et al.,* 2019). Salinity is another type of stress and a major

barrier to rice production after water scarcity. Increasing rice production against the backdrop of depleting water resources and increasing soil salinity requires the adoption of agricultural management practices that attenuate the limitations associated with these stresses like poor seedling establishment, low nutrient use efficiency, and susceptibility to various pests and diseases while simultaneously maximising rice yield. There is hence a need to develop low-cost farmer friendly technology for management of these stresses encountered by rice. In this context, microbe assisted crop production assumes prominence (Hohmann *et al.,* 2020) as it is an environment friendly technology based on the microorganisms that possess traits to encourage plant growth and alleviate plant stresses.

Methylotrophs, especially the pink pigmented facultative methylotrophic bacteria (PPFM) are one such type of beneficial bacterial community that assists plants in a variety of ways that improve the availability of nutrients like phosphorus, potassium zinc and iron, enhance plant growth and development and assist in mitigating the adverse effects of stress through insoluble mineral solubilizations, phytohormone production and synthesizing ACC deaminase enzyme. Probably due to these traits, the inoculation of *Methylobacterium* onto plants results in a growth promotion effect, as reported for rice (Tani *et al*., 2015, Lai *et al*., 2020) and also in maize (Vedavani *et al*., 2021) PPFM comes under the genus *Methylobacterium* and are strictly aerobic, Gram-negative, rods capable of growing on C_1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substances (Raghavendra and Santhosh 2019). The purpose of this study was to isolate and identify PPFM bacteria which possess multiple plant growth promoting and abiotic stress tolerance traits, and to identify through an in vitro study, the best isolates that improve rice seed germination and seedling growth. Thus, we screened PPFM bacteria isolated from field-growing rice plants for various beneficial traits related to the categories of plant growth promotion and abiotic stress tolerance so that they can be used as bioinoculants to improve plant growth.

MATERIAL AND METHODS

A. Isolation and characterization of PPFM isolates

PPFM bacteria were isolated by leaf imprinting and serial dilution technique (Raghavendra and Santhosh 2019) and putatively identified as PPFM based on growth on ammonium mineral salts (AMS) agar media with 0.5% methanol. The isolates were purified and maintained on AMS agar for further study. Morphological and biochemical characterization of the isolates were carried out by standard procedures.

B. Assessment of PPFM isolates for PGPR characteristics

All the isolates were screened for quantitative production of indole acetic acid (Gorden and Weber 1951). Culture supernatant of PPFM isolates grown for 7 days at 28°C in tryptone soya broth amended with tryptophan was used to assay for the production of IAA using Salkowski reagent and the development of pink colour was considered as positive for IAA. The intensity of pink colour was read in a spectrophotometer (UH 5300, Hitachi) at 540 nm. Based on the concentration of IAA produced, the bacterial isolates were classified into 4 groups. A score of 1 was assigned to isolates showing weak production (1.0 – 4.0 µg/ml IAA production), 2 to isolates with moderate production (4.0 – 7.0 µg/ml IAA production), 3 to isolates with high production (7.0 – 10.0 µg/ml IAA production). Isolates that did not produce IAA had a score of 0.

For gibberellic acid (GA) production, the isolates were grown in AMS broth for 7 days at 28°C. Gibberellic acid was extracted from the bacterial culture supernatant by the method described by Holbrook (1961) and the GA concentration was estimated at 254 nm using a Uv-Vis spectrophotometer (UH 5300 Hitachi). A score of 1 was assigned to isolates showing weak production (0.0 – 4.0 µg/ml GA production), 2 to isolates with moderate production (4.0 – 8.0 µg/ml GA production), 3 to isolates with high production (8.0 – 12.0 µg/ml GA production). Isolates that did not produce GA had a score of 0.

PPFM strains were screened for cytokinin production by using a bioassay method modified from Fletcher and McCuliagh, (1971). The assay was based on the ability of cytokinin to stimulate greening in etiolated cucumber cotyledons. Isolates were grown on one half of 0.6% minimal medium agar plate for 96 h at 30°C and ten etiolated cucumber cotyledons were placed 2mm apart from each other on the other half of the PPFM culture plate and incubated under green light (intensity of 55 µmol m^{-2} s^{-2}) for 3h. Minimal medium without culture was used as control. After incubation, the chlorophyll was extracted from cotyledons grown on different methylobacterial culture plates and control with acetone, and the concentration of the extracted chlorophyll was estimated from the absorbance of the extract measured at 665 nm using spectrophotometer. The cultures showing chlorophyll content ranging 1.000-1.166 mg g $tissue^{-1}$ were assigned a score of 1, cultures showing chlorophyll content ranging 1.166-1.332 mg g $tissue^{-1}$ were assigned a score of 2, and cultures showing chlorophyll content ranging 1.332-1.498 mg g $tissue^{-1}$ were assigned a score of 3.

The strains were screened for solubilization of phosphorus (Pikovskaya's, 1948) and the zone of solubilization formed around the colonies was recorded and Solubilizing Index (S.I) was calculated using the formula

$$S.I = C + Z / C$$

where,

Z = Solubilization zone (mm)

C = Colony diameter (mm)

Based on solubilization index the isolates were assigned scores. The isolates with SI values between 0-1 were assigned a score of 0, while SI values of 1-2, 2-3 and above 3 were given scores of 1, 2 and 3 respectively.

The solubilization of zinc might limit the growth of the bacteria at higher level. Unless the cultures tolerate a higher level of zinc its solubilization may not continue. Therefore, the ability of selected isolate to tolerate solubilized zinc was determined under in vitro condition

in nutrient broth containing different concentrations of soluble zinc ($ZnSO_4$.

The strains were tested for zinc solubilisation potential using tris minimal agar media (Saravanan *et al*., 2004) and based on the zone of solubilization formed around the colonies SI was calculated and the scores were assigned to isolates as described for phosphorus solubilization. Potassium solubilization index was also calculated and scores were assigned to isolates in a similar manner after screening for potassium solubilization on Aleksandrov's medium (Hu *et al*., 2006).

For detection of siderophore production by isolates, the chrome azurol sulfonate (CAS) agar media was used and the diameter of yellow to orange coloured zone around the isolates indicating siderophore production was recorded. Siderophore production index was calculated using the same formula as that of phosphate solubilization and the isolates with index range from 1.0 – 2.0 were given score 1, index from 2.0 – 3.0 were given score 2, index from 3.0 and above were given score 3.

HCN production was tested by growing PPFM cultures on AMS media plates (Castric and Castric 1983) supplemented with glycine (4.4g/l). A disc of Whatmann filter paper no.1 was sterilized and soaked in 0.5 % picric acid and 1% sodium carbonate and placed in the upper lid of the inoculated petri plates. The change in colour of the filter paper from yellow to light brown, brown or strong reddish brown, was taken as an indication of HCN production. Scores of 0 - 4 were assigned to isolates on the intensity of filter paper colour.

The bacterial strains were screened for their ability to utilize the 1-aminocyclo propane-1-carboxylate (ACC) as sole nitrogen source, a trait that is a consequence of the activity of the enzyme was ACC deaminase (Penrose and Glick 2003). Growth on plates having ACC as sole source of nitrogen is taken as an indicator of their ability of the isolate to produce enzyme ACC deaminase. The PPFM isolates were classified into 3 groups based on the growth in plates *viz*., + (less growth), ++ (moderate growth), +++ (high growth) and the scores were given as 1, 2 and 3 respectively.

C. Assessment of PPFM isolates for abiotic stress tolerance

The salt tolerance of PPFM isolates was tested by growing the cultures in AMS medium plates supplemented with 1, 2, 3, and 4% concentrations of NaCl (Ahlawat *et al.,* 2018) and the growth of isolates were examined after incubation of the plates for 48 h at 28 °C. Scores were assigned to the isolates based on growth characteristics on salt supplemented media. Moisture stress tolerance of isolates was determined by screening on AMS liquid media containing PEG 6000 at appropriate concentrations to make -0.05 MPa, -0.15 MPa, -0.30 MPa and -0.73 MPa (Kumar *et al.,* 2017) and after incubation at 28 °C for 24 h, growth was estimated by measuring the optical density at 600 nm by using a spectrophotometer. Growth at -0.73 MPa was used as the criterion to select strains tolerant to water deficit stress (Jorge *et al*., 2019). Based on the growth of isolates at -0.73 MPa, scores 3, 2, 1 and 0 were assigned to isolates with high, moderate, low and no growth respectively.

D. In vitro seed germination assay of rice

A loopful of each bacterial culture was inoculated individually in AMS medium and allowed to grow for 3-5 days. The cultures were then centrifuged and the pellet obtained was washed with phosphate saline buffer (PBS), dissolved in 25 ml of PBS to obtain an OD of 1 at OD_{600} nm which is equivalent to 1×10^8 CFU/ml. The rice seeds (DRR Dhan 46) were surface sterilized with 0.15% $HgCl_2$ for 1 min, followed by 70% ethanol for 1min and finally washed with distilled water. The seeds (25 seeds per bacterial treatment) were soaked in different cultures and incubated overnight. The overnight soaked seeds were placed on water agar and allowed to germinate for 7 days at 27°C. The data such as germination percentage, root length, shoot length were measured and vigour index calculated (Shende *et al.,* 1977).

Seedling vigour index = (Root length + Shoot length) X Germination percentage.

The PPFM cultures inducing vigour index ranging between 1000-1300 in rice seedlings were assigned a score of 1, while the ranges between 1400-1600 and 1700-1900 were assigned a score of 2 and 3 respectively.

E. Scheme for the selection of promising PPFM isolates

The most efficient PPFM strains were selected using a bonitur scale as described by Padda *et al.* (2021) and Krechel *et al.* (2002). Isolates tested in this study were ranked based on the results of different plant growth promoting mechanisms. In this scale, scores were given to PPFM strains for each *in vitro* plant growth promoting trait examined with a highest possible score of 36 points (for 12 traits* 3 maximum score per trait). Based on the ranking, five PPFM isolates with the highest scores will be selected as the most efficient PPFM isolates (Scoring explained in Fig. 6)

F. Identification of isolates by 16S rDNA sequence analysis

Genomic DNA from the highest scoring methylobacteria (5 isolates) were isolated using Gsure bacterial RNA isolation kit (GCC Biotech, India) following the manufacturer's protocol. Gene amplification of 16S rRNA was carried out in BioRad T100 thermocycler (BioRad, USA) using two primer sets (Srija *et al*., 2022) *viz*, were i) 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3') and ii) 785F (5'-GGATTAGATACCCTGGTA-3') and 1492R (CGGTTACCTTGTTACGACTT-3'). The 20 µl PCR reaction mixture (bacterial DNA- 2 µl, dNTPs- 1.6 µl, 10X buffer- 2µl, Taq polymerase- 0.2µl) was subjected to an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min for amplification of 16S rRNA genes. The amplified DNA after sanger sequencing were aligned and merged contigs were created using Cap3 Bioedit Software. The contigs were compared against the sequences of 16S rRNA of bacterial isolates available in the National Centre for

Biotechnology Information (NCBI) Nucleotide Database (http://www.ncbi.nih.gov/blast) and the isolated bacteria were identified based on maximum percentage of similarity of the sequences.

RESULTS AND DISCUSSION

A. Isolation of pink pigmented facultative methylotrophs

The PPFM'S were isolated from the rice grains, the surfaces of leaf samples and rhizosphere soils of rice. Nineteen pink pigmented facultative methylotrophs were isolated by leaf imprinting method and serial dilution method using AMS medium which was a selective medium for isolating PPFM'S (Lidstorm and Chistoserdova 2002).

Out of nineteen PPFM isolates, seventeen isolates are obtained from phyllosphere (Table 1, Fig. 1). It has been reported that 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 (Trotsenko *et al.,* 2001).

Several authors have previously reported the natural association of PPFMs with various plants. Madhaiyan *et al.* (2007) isolated a pink pigmented facultatively methylotrophic bacterial strain CBMB20^T from the stem tissues of rice (*Oryza sativa* L. 'Dong-Jin'). Chaudhry *et al.* (2016) isolated two strains of *Methylobacterium* species from rice seeds while screening for endophytic bacteria.

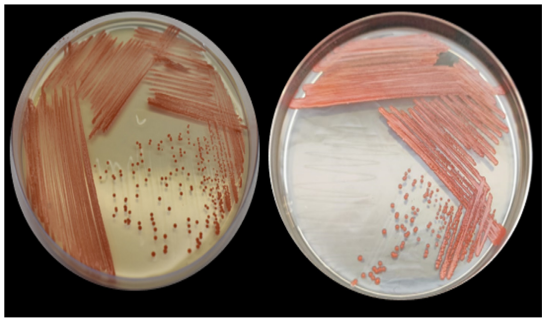

Fig. 1. Purified PPFM cultures on AMS media.

Table 1: Sources of PPFM isolates from rice growing areas of Telangana region.

Sr. No.	Isolate code	Isolation source (Rice genotype)	Habitat	Place	GPS Location
1	IIRRSV22-1	Akshaya dhan	Phyllosphere	Patancheru	17.5111 N, 78.2752 E
2	IIRRSV22-2	Purple puttu	Phyllosphere	Patancheru	17.5111 N, 78.2752 E
3	IIRRSV22-3	Akshaya dhan	Phyllosphere	Patancheru	17.5111 N, 78.2752 E
4	IIRRSV22-4	N-22	Phyllosphere	Patancheru	17.5111 N, 78.2752 E
5	IIRRSV22-5	Sonamasuri	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
6	IIRRSV22-6	JGL-11118	Phyllosphere	College farm, Rajendranagar	17.3220 N, 78.4023 E
7	IIRRSV22-7	JGL-11118	Phyllosphere	College farm, Rajendranagar	17.3220 N, 78.4023 E
8	IIRRSV22-8	JGL-11118	Phyllosphere	College farm, Rajendranagar,	17.3220 N, 78.4023 E
9	IIRRSV22-9	N-22	Seed endosphere	ICAR-IIRR	17.3201 N, 78.3939 E
10	IIRRSV22-10	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
11	IIRRSV22 -11	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
12	IIRRSV22 -12	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
13	IIRRSV22-13	Akshaya dhan	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
14	IIRRSV22-14	Purple puttu	Rhizosphere	ICAR-IIRR	17.3201 N, 78.3939 E
15	IIRRSV22-15	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
16	IIRRSV22-16	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E
17	IIRRSV22-17	JAK 685 15116	Phyllosphere	Rajendra nagar	17.3223 N, 78.3993 E
18	IIRRSV22-18	RNR 21278	Phyllosphere	Rajendra nagar	17.3223 N, 78.3993 E
19	M1	Purple puttu	Phyllosphere	ICAR-IIRR	17.3201 N, 78.3939 E

B. Morphological and biochemical characterization

All PPFM isolates obtained in this study had pink colonies on ammonium mineral salts medium (AMS) with varying intensities of pigmentation ranging from light to dark pink, allowing them to be distinguished from other methylotrophic organisms found on plant surfaces (Table 2). A series of biochemical tests were used to further characterise the PPFM isolates. Some of the isolates were positive for oxidase, catalase and urease activity (Table 2). Similar results were documented by Nysanth *et al.,* (2019) in which five PPFM isolates from paddy were positive for oxidase, urease, catalase and indole production and similar results were also observed by Lakshmi *et al*., (2012) in which the biochemical analysis of the PPFM isolates from the phyllosphere region of cotton revealed that they are positive for catalase, oxidase and urease (Fig. 2).

Table 2: Morphological and biochemical characteristics of pink pigmented facultative methylotroph isolates.

Sr. No.	Isolate code	Cell shape	Gram reaction	Pigmentation	Biochemical characterization		
					Catalase	Oxidase	Urease
1	IIRRSV22-1	Rod	Negative	Dark pink	Positive	Positive	Positive
2	IIRRSV22-2	Rod	Negative	Light pink	Positive	Positive	Positive
3	IIRRSV22-3	Rod	Negative	Light pink	Positive	Positive	Positive
4	IIRRSV22-4	Rod	Negative	Dark pink	Positive	Positive	Positive
5	IIRRSV22-5	Rod	Negative	Light pink	Positive	Positive	Positive
6	IIRRSV22-6	Rod	Negative	Light pink	Positive	Positive	Positive
7	IIRRSV22-7	Rod	Negative	Pale pink	Positive	Positive	Positive
8	IIRRSV22-8	Rod	Negative	Dark pink	Positive	Positive	Positive
9	IIRRSV22-9	Rod	Negative	Dark pink	Positive	Positive	Positive
10	IIRRSV22-10	Rod	Negative	Pale pink	Negative	Positive	Positive
11	IIRRSV22-11	Rod	Negative	Light pink	Positive	Positive	Negative
12	IIRRSV22-12	Rod	Negative	Pale pink	Positive	Negative	Positive
13	IIRRSV22-13	Rod	Negative	Dark pink	Positive	Positive	Positive
14	IIRRSV22-14	Rod	Negative	Dark pink	Negative	Positive	Negative
15	IIRRSV22-15	Rod	Negative	Pale pink	Positive	Negative	Negative
16	IIRRSV22-16	Rod	Negative	Light pink	Positive	Positive	Positive
17	IIRRSV22-17	Rod	Negative	Pale pink	Positive	Positive	Positive
18	IIRRSV22-18	Rod	Negative	Light pink	Positive	Positive	Positive
19	M1	Rod	Negative	Dark pink	Positive	Positive	Positive

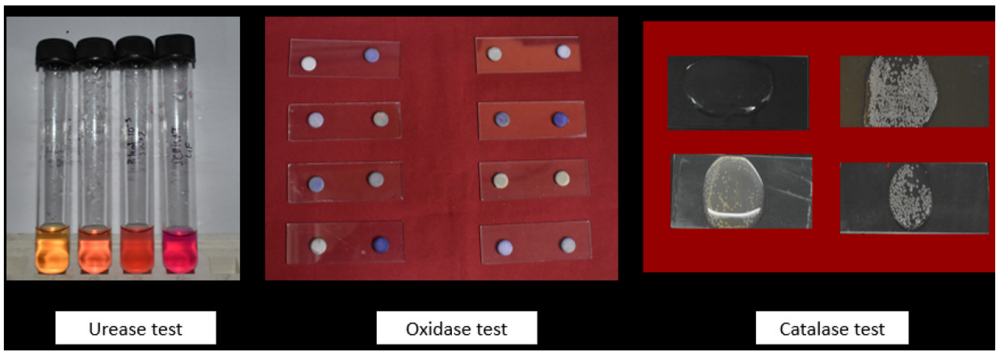

Fig. 2. Biochemical characteristics of PPFM isolates.

C. Screening of the PPFM isolates for PGPR traits

Methylotrophs can aid in plant growth and development through various mechanisms like synthesising compounds that promote plant growth, increase nutrient uptake, and also by acting as biocontrol agents. All the nineteen PPFM isolates were evaluated for multiple PGPR traits (Fig. 3).

Indole-3-acetic acid (IAA) is the most common naturally occurring plant hormone. All 19 isolates produced pink to red colour with variation in intensity when tested using Salkowski's reagent. The isolates showed wide variation in IAA synthesis ranging from 1.75 to 10.88 µg ml^{-1} of culture filtrate and the results are presented in Table 3. Maximum IAA production was observed in IIRRSV22-5 whereas the minimum production was observed in IIRRSV22-16. Jones (2010) observed variability among PPFM isolates in producing IAA ranging from 0.14 to 25.12 µg ml^{-1}. Similarly, Yim *et al*., 2010 carried out quantitative analysis of IAA using Salkowski reagent from culture liquids of the *Methylobacterium* strains in the presence of L-tryptophan and obtained 2.33 and 4.03 µg IAA ml^{-1} respectively after 5 days of inoculation.

The highest GA production (Table 3) among the isolates was recorded in IIRRSV22 -5(10.20 µg/ml) whereas the lowest was observed in IIRRSV22-13(02.02 µg/ml).

Similar variation among isolates in the production of GA was reported by Savitha *et al.*, 2019 in chilli with the GA content ranging from30.10µg/ml to 128.18µg/ml. The results are also consistent with that of Anu, 2003, wherein the production of gibberellic acid by eight of the PPFM isolates tested varied from 10.9 µg/ml to 106.97 µg/ml.

Cytokinins are adenine derivative phytohormones that control cell division, cell cycle and stimulate developmental processes in plants (Srivastava, 2002). An assay based on the ability of cytokinin to stimulate greening in etiolated cucumber cotyledons was used in this study to identify the PPFM isolates that produced this hormone. The isolates IIRRSV22-5 (1.499 mg g tissue^{-1}), IIRRSV22-9 (1.305 mg g tissue^{-1}), IIRRSV22-1(1.250 mg g tissue^{-1}), IIRRSV22-4 (1.278 mg g tissue^{-1}) and M1 (1.328 mg g tissue^{-1}) were able to induce higher chlorophyll production in cotyledons (Table 3, Fig. 3) compared to remaining PPFM isolates and control (1.005 mg g tissue^{-1}). Similar results were observed by Hussain and Hasnain, 2009 in which greening occurred in cotyledons when shifted to green light from dark for 3 h. They also observed enhanced chlorophyll formation (73.08, 61.54 and 51.28%) in etiolated cucumber cotyledons exposed to *Bacillus licheniformis* Am2, *Bacillus subtilis* BC1 and *Pseudomonas aeruginosa* E2 respectively against control.

Table 3: Indole acetic acid, Gibberellic Acid, Cytokinin production and ACC deaminase activity of PPFM isolates.

Sr. No.	Isolate	IAA(µg/ml)	GA(µg/ml)	Chlorophyll content (mg g tissue^{-1})	ACC deaminase activity
1.	IIRRSV22-1	05.08	04.92	1.250	+
2.	IIRRSV22-2	02.42	03.00	1.218	-
3.	IIRRSV22-3	07.58	07.12	1.400	+
4.	IIRRSV22-4	04.71	04.77	1.278	+
5.	IIRRSV22-5	10.88	10.20	1.498	++
6.	IIRRSV22-6	02.79	-	1.034	+
7.	IIRRSV22-7	03.25	-	1.006	+
8.	IIRRSV22-8	02.37	02.54	1.210	+
9.	IIRRSV22-9	04.71	-	1.305	-
10.	IIRRSV22-10	03.75	02.69	1.111	-
11.	IIRRSV22-11	04.12	-	1.152	-
12.	IIRRSV22-12	02.83	-	1.220	-
13.	IIRRSV22-13	02.70	02.02	1.197	+
14.	IIRRSV22-14	03.04	-	1.110	-
15.	IIRRSV22-15	02.12	-	1.040	+
16.	IIRRSV22-16	01.75	-	1.045	-
17.	IIRRSV22-17	04.62	-	1.065	-
18.	IIRRSV22-18	02.71	-	1.034	-
19.	M1	06.12	05.14	1.328	++
Control	-	-	-	1.005	-

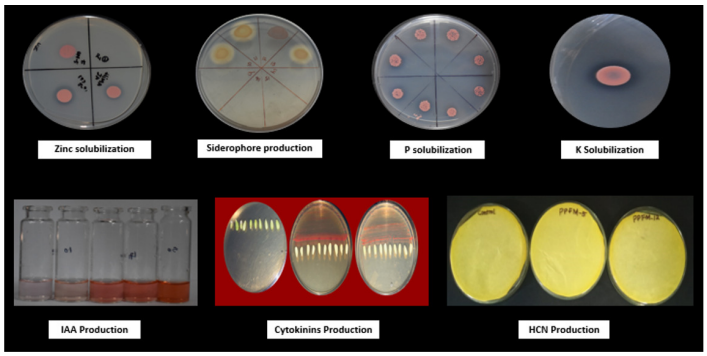

Fig. 3. Evaluation of PPFM isolates for multiple PGPR traits.

Phosphorus (P) is a major essential macronutrient for plant growth and development. *Methylobacterium* spp. are able to dissolve inorganic phosphates, which in turn promotes phosphate uptake and metabolism in plants. (Rodriguez *et al*., 2006). The isolated PPFM cultures were tested for phosphorus solubilisation on Pikovskaya's agar. Among nineteen isolates, IIRRSV22-9 showed the maximum solubilization index (3.12) followed by IIRRSV22-1 with solubilisation index of 2.8 (Table 4, Fig. 3) and lowest solubilisation index of 2.77 was recorded in IIRRSV22-15 while the remaining isolates had SI ranging from 2.5 to 3.0. In a similar study, thirteen pink pigmented facultative methylotrophic strains from Adyar and Cooum rivers in Chennai and

forest soil samples in Tamil Nādu, along with *Methylobacterium extroquens*, *M. organophilum*, *M. gregans*, and *M. komagatae* were screened for phosphate solubilization in plates and P-solubilization index ranging from 1.1-2.7 have been reported by the authors (Jayashree *et al.*, 2011). Savitha *et al.* (2020) have also reported similar findings with 8 pink pigmented facultative methylotrophic bacteria isolated from the chilli grown in north Karnataka.

Zinc solubilizing bacteria are potential alternatives for zinc supplementation as they convert insoluble zinc to plant available forms. Isolates were tested for zinc solubilisation using Tris minimal media. Out of 19 isolates,16 isolates have shown zone of solubilisation (Table 4, Fig. 3). IIRRSV22-5 showed the maximum solubilisation index (3.5) and the least was observed in IIRRSV22-17 (2.33). Vadivukkarasi *et al.* (2020) assessed the zinc solubilizing ability of *Methylobacterium komagatae* sp. IISRGPPFM13 strain using Tris minimal media amended with ZnO and observed a dissolution zone of 1.3 cm with a solubilization index of 4.9. Similar findings were reported by Verma *et al.* (2014), wherein all the methylotrophic isolates had SI which ranged from 3.4-3.6.

Potassium (K) is considered a major and essential plant macronutrient for biological growth and development. The potassium-solubilizing microorganisms solubilize the insoluble potassium to plant available soluble forms improving plant growth and yield. Potassium solubilisation was observed only in IIRRSV22-8 and zone of solubilisation was 3 (Table 4, Fig. 3). Similar findings were reported by Prajapati *et al.* (2017) when native phyllospheric methylotrophic isolates and their consortium were tested for their potash solubilizing efficiency on Alendreskov's media containing mica as natural 'K' substrate. The diameter of potash solubilization zone on Alendreskov's mica media plates reported by them were between 3-4 mm for isolates M 10, M-15, *B. aerius* AAU M 8 and B. *megaterium* AAU M 29.

HCN works by inhibiting the enzymes that have metal ion cofactor such as Cu^{2+} and plays a role in the biological control of disease -causing organisms. The isolates were tested for the production of HCN in glycine supplemented media but none of the isolates were confirmed as cyanogenic due to their inability to cause colour changes on indicator filter paper. Raghavendra and Santhosh (2019) have also observed that the PPFM isolates from major rice growing areas of Hyderabad and Karnataka were negative for HCN production (Fig. 3).

Siderophore production by PPFM isolates was tested using by spotting all the nineteen isolates on CAS agar plates. The ability of siderophore production was limited to only some isolates as observed by the formation of orange colour zones around bacterial colonies. Out of nineteen isolates, nine isolates were found to be positive (IIRRSV22-1,3,4,5,9,10,11,13 and M1) for siderophore production and the results are presented in Table 4 and Fig. 3. The results are confirmative with the findings of Lacava *et al.*, (2008) who observed the production of siderophores by 37 endophytic *Methylobacterium* sp. strains from citrus plants. Similar reports were presented by Kassem *et al.* (2013) in which all the 12 PPFMs isolated from the clay soil were positive for the siderophore production. They reported that the highest siderophore production was in *Methylobacterium rhodinum and Methylobacterium aminovorans* cultures.

Plant growth promoting methylotrophic bacteria which possess the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, promote plant growth and development by decreasing the levels of stress ethylene in plants (Glick *et al.*, 1998). All the PPFM isolates were found to grow on plates containing $(NH4)_2SO_4$ as nitrogen source while only a few isolates grew on plates having ACC as sole source of nitrogen demonstrating their ability to produce the enzyme ACC deaminase. The isolates that showed growth on plates having ACC as sole source of nitrogen were IIRRSV22-1, IIRRSV22-3, IIRRSV22-4, IIRRSV22-5, IIRRSV22-8, IIRRSV22-13, IIRRSV22-15 and M1 isolates. (Table 3). Similar findings were presented by Chinnadurai *et al.* (2009) in which the presence of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity among the phyllosphere methylobacteria of rice. Yim *et al.*, (2010) reported that *M. oryzae* strains CBMB20 and CBMB110 possessed ACC deaminase activity of 94.48 and 24.74 nmol α-ketobutyrate mg^{-1} protein h^{-1} respectively.

D. Screening for abiotic stress tolerance

Salt stress. The methylobacterial isolates were tested for salt tolerance in AMS medium supplemented 1-4% of NaCl. While all the isolates (IIRRSV22-1 to IIRRSV22-19) showed growth on medium containing 1% NaCl. Higher levels of NaCl repressed the growth of PPFM isolates. No isolate showed tolerance against 4% NaCl and the results are presented in Table 5. The results showed similarity with the work of Ahlawat *et al.*, 2018 in which out of the 60 methanol utilizing methylobacteria isolated from rhizosphere, nodule and phyllosphere of different crops, only 10 bacterial isolates showed tolerance against 4% NaCl concentration (Fig. 4).

Moisture stress. Effect of moisture stress (PEG 6000) on growth of PPFM isolates was evaluated at different concentrations i.e., -0.05 MPa, -0.15 MPa, -0.30 MPa, -0.73 MPa. All isolates showed growth at -0.05 MPa, -0.15 MPa, and -0.30 MPa while only six of the isolates (IIRRSV22-1,3,4,5,8 and M1) showed growth at - 0.73 MPa. The data on PPFM isolates growth is expressed in terms of optical density OD_{600} at different PEG concentrations (Table 5). In a similar study, Jorge *et al.*, 2019 assessed *Methylobacterium* tolerance to drought conditions by growing the strains in minimal medium supplemented with PEG 6000 at different concentrations (0%, 5%, 10%, 15% and 20%) and the isolates with highest O.D. values at 20% PEG concentration were considered as potential plant growth promoters under water deficit conditions.

Table 4: Phosphate potassium and zinc solubilisation index and siderophore production index of PPFM isolates.

Isolates	Solubilization index			Siderophore Production index
	P	K	Zn	
IIRRSV22-1	2.80	-	2.80	3.42
IIRRSV22-2	-	-	3.10	-
IIRRSV22-3	3.10	-	3.20	2.88
IIRRSV22-4	3.10	-	3.14	2.78
IIRRSV22-5	2.90	-	3.50	3.57
IIRRSV22-6	-	-	2.57	-
IIRRSV22-7	-	-	-	-
IIRRSV22-8	-	3.00	2.42	-
IIRRSV22-9	3.12	-	3.12	2.71
IIRRSV22-10	-	-	2.80	2.62
IIRRSV22-11	-	-	2.85	1.85
IIRRSV22-12	-	-	2.60	-
IIRRSV22-13	-	-	2.50	2.37
IIRRSV22-14	-	-	2.42	-
IIRRSV22-15	2.77	-	3.10	-
IIRRSV22-16	-	-	-	-
IIRRSV22-17	-	-	2.33	-
IIRRSV22-18	-	-	-	-
M1	3.11	-	3.33	3.25

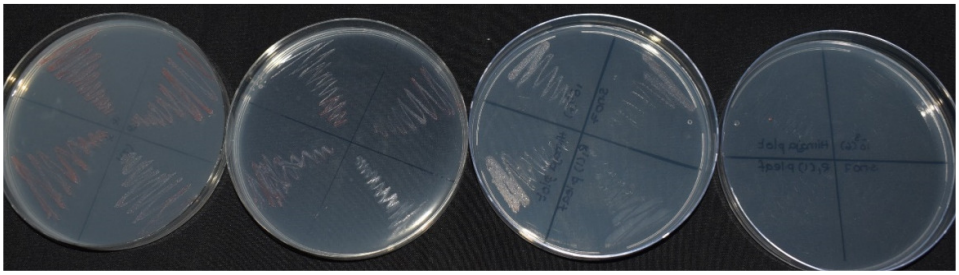

Fig. 4. Evaluating PPFM isolates for salt stress tolerance.

Table 5: Growth of PPFM isolates under salt and water stress.

Isolate	NaCl concentration (%)				PEG concentration			
	1%	2%	3%	4%	-0.05 M Pa	-0.15 M Pa	-0.30 M Pa	-0.73 M Pa
IIRRSV22-1	+	+	+	-	1.45	1.14	0.13	0.03
IIRRSV22-2	+	+	-	-	0.56	0.28	0.05	-
IIRRSV22-3	+	+	+	-	1.49	1.27	0.24	0.02
IIRRSV22-4	+	+	+	-	0.53	0.37	0.09	0.04
IIRRSV22-5	+	+	+	-	1.50	0.74	0.43	0.04
IIRRSV22-6	+	+	-	-	0.46	0.29	0.07	-
IIRRSV22-7	+	+	-	-	0.53	0.37	0.14	-
IIRRSV22-8	+	+	-	-	0.63	0.17	-	-
IIRRSV22-9	+	-	-	-	0.47	0.25	0.07	0.03
IIRRSV22-10	+	+	-	-	0.55	0.14	0.09	-
IIRRSV22-11	+	+	+	-	0.67	0.11	-	-
IIRRSV22-12	+	+	-	-	0.43	0.21	0.07	-
IIRRSV22-13	+	+	+	-	0.72	0.39	0.04	-
IIRRSV22-14	+	+	+	-	0.62	0.08	0.03	-
IIRRSV22-15	+	-	-	-	0.51	0.35	0.07	-
IIRRSV22-16	+	+	-	-	0.23	0.13	-	-
IIRRSV22-17	+	+	-	-	0.44	0.72	0.32	-
IIRRSV22-18	+	+	-	-	0.34	0.34	0.08	-
M1	+	+	+	-	0.79	0.45	0.28	0.03

E. In vitro germination assay

Seed priming by pink pigmented facultative methylotrophic bacteria has shown positive effect on germination and development of rice variety DRR Dhan 46 (Fig. 5). All the PPFM isolates showed increase in seed germination rate when compared with control. The lowest germination rate was observed in seeds treated with IIRRSV22-8 (92%) and untreated control (90%) while the treatments with all the other isolates showed 100 percent germination. Among all the treatments, IIRRSV22-5 showed significantly higher (Table 6) root and shoot length (12.47cm and 6.49 cm) compared to

IIRRSV22 -3(12.61 cm and 6.27 cm), IIRRSV22-1 (10.63 cm and 5.97 cm), IIRRSV22-4 (10.37 and 6.20 cm), IIRRSV22-11 (9.88cm and 5.99 cm) and M1 (10.24 cm and 5.53 cm).

Pertaining to the seedling vigour index (SVI), the highest SVI was observed in IIRRSV22-5(1896.0) followed by IIRRSV22 -3, IIRRSV22-1, IIRRSV22-4, IIRRSV22-11 and M1 (1888.0, 1666.0, 1657.0, 1587.0 and 1577.0) respectively compared to control (1280.0).

The results demonstrated that the seeds treated with PPFM isolates enhanced seedling growth parameters, suggesting that the bacterial inoculation shortens the time needed for germination as it took only 3 days when compared with control (4 days). These findings support the hypothesis that one or more PGPR characteristics of pink pigmented facultative methylotrophic bacterial isolates could have contributed to the overall improvement in rice seed germination and seedling growth. IAA is an important regulator of many biological processes like cell division, elongation and differentiation. Auxins also promote root growth, which increases the surface area available for nutrient and water uptake which impacts directly on seedling development. In addition, the production of siderophores and stress relieving enzymes like ACC deaminase by some of the isolates too could have contributed to higher seed germination and growth.

Similar results were reported by Krishnamoorthy *et al.* (2018) who isolated 80 plant growth promoting phyllospheric methylotrophic isolates from Tamil Nādu. *Invitro* germination assay revealed that the methylotrophic isolates enhanced root growth and improved formation of root hairs, with the highest radicle length and shoot length observed in the seeds treated with *M. radiotolerans*. Madhaiyan *et al.* (2004) observed the plant growth promoting activity of methylotrophic bacteria under greenhouse conditions in which *Methylobacterium* inoculation of rice seeds proved to be beneficial in improving the rate of germination and seedling vigour index.

F. Criteria for selection of promising isolates

A bonitur scale was generated based on the scores assigned to each trait to select the most promising pink pigmented facultative methylobacteria. Five isolates with the maximum score were selected. The five selected isolates were IIRRSV22-1, IIRRSV22-3, IIRRSV22-4, IIRRSV22-5 and M1 with the scores of 21, 25, 23, 27 and 25 respectively (Table 7, Fig. 6).

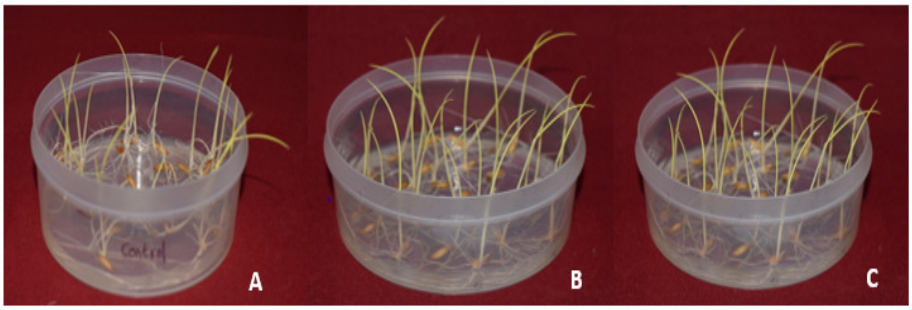

Fig. 5. In vitro evaluation of rice seeds treated with PPFM isolates (A-Control, B-IIRRSV22-5, C-M1).

Table 6: Effect of PPFM treatment on germination and growth of rice seedlings.

Isolates	Germination percentage (%)	Root length(cm)	Shoot length(cm)	Seed vigour index
IIRRSV22-1	100	10.63	5.97	1666
IIRRSV22-2	100	09.05	5.46	1451
IIRRSV22-3	100	12.61	6.27	1888
IIRRSV22-4	100	10.37	6.20	1657
IIRRSV22-5	100	12.47	6.49	1896
IIRRSV22-6	100	07.15	5.40	1255
IIRRSV22-7	100	10.37	6.20	1657
IIRRSV22-8	92	08.25	5.94	1419
IIRRSV22-9	100	09.54	4.57	1411
IIRRSV22-10	100	07.67	5.83	1350
IIRRSV22-11	100	09.88	5.99	1587
IIRRSV22-12	100	08.77	6.27	1504
IIRRSV22-13	100	09.65	5.65	1530
IIRRSV22-14	100	09.47	5.67	1514
IIRRSV22-15	100	09.56	5.43	1499
IIRRSV22-16	100	08.03	5.13	1316
IIRRSV22-17	100	09.00	5.45	1445
IIRRSV22-18	100	8.93	5.07	1400
M1	100	10.24	5.53	1577
Control	90	8.85	3.95	1252
CD (0.05)	4.42	0.94	0.56	150.52
CV%	2.70	6.00	6.09	6.02

Table 7: Assessment and ranking of PPFM isolates on their ability to function as PGPR and promote the growth of *Oryza sativa*.

Isolate code	Solubilization			IAA	GA	CK	HCN	Sid	ACCD	Salt stress	Moisture stress	SVI	Bonitur scale	Rank
	P	Zn	K											
IIRRSV22-1	2	2	0	2	2	2	0	3	1	3	2	2	21	3rd
IIRRSV22-2	0	3	0	1	1	2	0	0	0	2	0	2	11	8th
IIRRSV22-3	3	3	0	3	3	3	0	2	1	3	1	3	25	2nd
IIRRSV22-4	3	3	0	2	2	2	0	2	1	3	3	3	23	4th
IIRRSV22-5	2	3	0	3	3	3	0	2	2	3	3	3	27	1st
IIRRSV22-6	0	2	0	1	0	1	0	0	1	2	0	1	8	12th
IIRRSV22-7	0	0	0	1	0	1	0	0	1	2	0	3	8	12th
IIRRSV22-8	0	2	3	1	1	2	0	0	1	2	0	2	14	6th
IIRRSV22-9	3	3	0	2	0	2	0	2	0	1	2	2	17	5th
IIRRSV22-10	0	2	0	1	1	1	0	1	0	2	0	2	10	9th
IIRRSV22-11	0	2	0	2	0	1	0	1	0	2	0	2	10	9th
IIRRSV22-12	0	2	0	1	0	2	0	0	0	2	0	2	9	10th
IIRRSV22-13	0	2	0	1	1	2	0	2	1	2	0	2	13	7th
IIRRSV22-14	0	2	0	1	0	1	0	0	0	1	0	2	7	13th
IIRRSV22-15	2	3	0	1	0	1	0	0	1	1	0	2	11	8th
IIRRSV22-16	0	0	0	1	0	1	0	0	0	2	0	2	6	14th
IIRRSV22-17	0	2	0	2	0	1	0	0	0	2	0	2	9	10th
IIRRSV22-18	0	0	0	1	0	1	0	0	0	2	0	2	6	14th
M1	3	3	0	3	2	2	0	3	2	3	2	2	25	2nd

*P-Phosphorus, Zn-Zinc, K-Potassium, IAA-Indole Acetic Acid, GA-Gibberellic Acid, CK-Cytokinin, Sid-Siderophore, HCN-Hydrogen Cyanide, SVI-Seed vigour index

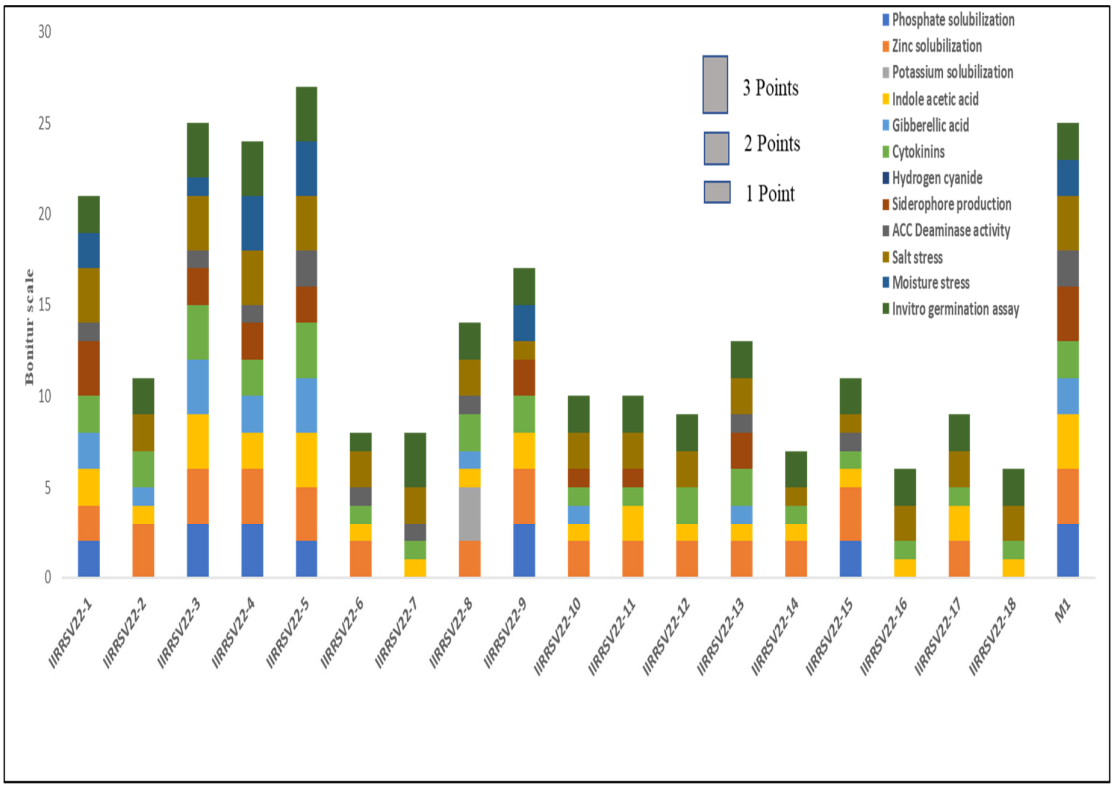

Fig. 6. Scoring of PPFM isolates based on plant growth promoting traits, abiotic stress tolerance and invitro germination assay.

G. Molecular identification of bacterial isolates

The assembled 16S rRNA gene sequences of approximately 1500 bp, obtained after amplification and sequencing, were used to identify the selected five isolates by searching for homology against the National Center for Biotechnology Information (NCBI) non- redundant nucleotide sequence database using Basic Local Alignment Search Tool (BLAST). Based on the homology of the sequences to that in the NCBI, the molecular identity of the bacterial isolates were established. The identity of the isolates is shown (Table 8) along with the accession numbers of 16S rRNA gene sequences that have been deposited in the National Center for Biotechnology Information database.

The PPFM isolates identified in this study have also been reported earlier as possessing several plant growth promoting traits. *Methylobacterium aquaticum* which was first reported by Tani *et al.* (2012) in rice was found to produce siderophores. Priya *et al.* (2019) reported a significant increase in seed germination and seedling vigour in groundnut inoculated with *Methylobacterium radiotolerans* compared to uninoculated control. *Methylobacterium phyllosphaerae* was reported by Madhaiyan *et al.* (2009) as a potential plant growth promoting PPFM for rice plant. Sivakumar *et al.* (2017) in a study using tomato crop observed that foliar spraying of *Methylobacterium* sp could alleviate drought stress. Several *Methylobacterium* sp. have also been reported to possess the ability to solubilize phosphates and thus making the unavailable phosphorus available to growing plants. (Agafonova *et al*., 2013) while *Methylobacterium* sp CBMB12, CBMB27, CBMB15 and CBMB20 lowered ACC accumulation in tomatoes by 50% and promoted tomato growth by 50% to 80 % during stress (Madhaiyan *et al*., 2007a). *Methylobacterium fujisawaense* isolated from rice plants produced IAA, and was observed to increase the root and shoot lengths according to Lee *et al.* (2004).

Table 8: Molecular identification of the PPFM isolates based on 16s RNA gene.

Sr. No.	Isolate ID	Identified strain	Accession number
1	IIRRSV22-1	*Methylobacterium aquaticum*	OP748929
2	IIRRSV22-3	*Methylobacterium phyllosphaerae*	OP748930
3	IIRRSV22-4	*Methylobacterium radiotolerans*	OP748936
4	IIRRSV22-5	*Methylobacterium sp*	OP748943
5	M1	*Methylobacterium fujisawaense*	MF171122

CONCLUSION

PPFM isolates were screened PGP traits like phytohormone production, insoluble mineral solubilization, ACC deaminase enzyme activity and siderophore production. In addition, the abiotic stress tolerance ability of PPFM isolates under drought and salt stress conditions were also investigated. The PPFM isolates were also assessed for their ability to improve germination and seedling vigour of rice. Based on these investigations, the most promising isolates were identified as *Methylobacterium aquaticum*, *Methylobacterium radiotolerans, Methylobacterium* sp, *Methylobacterium fujisawaense*. The strains identified in this study seem to be ideal candidates for promotion as bio-inoculants, due to their multiple PGP traits, abiotic stress tolerance and ability to promote rice seedling growth and vigour.

FUTURE SCOPE

The developed PPFM cultures need to be evaluated on DSR under field conditions to corroborate the results of the present *in vitro* study and to assess their potential to promote growth and yield of rice under abiotic stresses.

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