

Screening of pink pigmented facultative methylotrophs for growth enhancement in paddy



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ABSTRACT

A pot culture experiment was conducted to study the effect of PPFMs isolated from the phyllosphere of paddy collected from different agro climatic conditions of Kerala by leaf imprint method on growth and yield of paddy using variety Jyothi (Ptb-39). Application of PPFM isolates significantly increased growth, biomass production and yield of paddy. PPFM11 treated plants recorded the highest grain yield of 46.30 g hill⁻¹ whereas the control recorded a grain yield of 33.65 g hill⁻¹. The reference culture recorded a grain yield of 38.40 g hill⁻¹. PPFM isolates significantly improved the chlorophyll content, cell membrane stability and proline content of the plant compared to untreated plants. The isolates PPFM11, PPFM16 and PPF19 were adjudged as superior isolates based on growth promotion efficacy and grain yield and yield attributes of paddy. These three isolates were identified as *Methylobacterium* spp. based on morphological, biochemical and molecular characteristics.

Plants produce several natural products which include carbon compounds that range in complexity from simple esters to structurally diverse compounds such as polyketides, carbohydrates, lignans, flavanoids, terpenoids, tannins, and alkaloids. Among these compounds methanol, one of the simplest organic molecules, is also a by-product of plant cell wall metabolism in rapidly growing plant organs. The methanol produced is emitted via stomatal pores of the epidermis. The plant phyllosphere supports a large and complex microbial community and bacteria are viewed to be the dominant microbial inhabitants of the phyllosphere. Mainly, leaves comprise a significant microbial habitat. The terrestrial leaf surface area that would possibly be colonized by microbes is over 6.4×10^8 km², which support bacterial population of about 10^{26} cells. Plant phyllosphere is an ecological niche which shelters highly abundant *Methylobacterium* species of 10^4 – 10^7 colony forming units (CFU) per leaflet (Mizuno et al., 2012).

Bacteria belonging to the genus *Methylobacterium* are commonly known as Pink-pigmented facultative methylotrophic bacteria. They are strict aerobes, Gram-negative, facultative methylotrophic rods. They are able to grow on C₁ compounds like methanol and methylamine and also on a variety of C₂, C₃, and C₄ compounds (Trotsenko et al., 2001).

These common prokaryotic epiphytes are classified within the α -Proteobacteria and distributed in various plants which include angiosperms, gymnosperms and even lower plants (Basile et al., 1969; Austin and Goodfellow, 1979; Corpe and Basile, 1982; Corpe, 1985). Several species of methylotrophic bacteria are found in association with terrestrial and aquatic plants, colonizing roots, leaf surfaces and growing buds (Trotsenko et al., 2001; Lidstrom and Chistoserdova, 2002).

They have been reported to produce plant growth regulators like zeatin and related cytokinins and auxins (Holland and Polacco, 1994), which have significant effect on seed germination and seedling growth. Production of gibberellic acid (GA) by *Methylobacterium* has already been reported (Thangamani and Sundaram, 2005). Additionally, *Methylobacterium* have been reported for the production of urease enzyme (Holland and Polacco 1994), vitamin B₁₂ production (Basile et al., 1985), nitrogen fixation and nodule formation (Raja et al., 2006), phosphate solubilization, synthesis of siderophores (Simionato et al., 2006) and for the existence and prevalence of ACC deaminase enzyme (Madhaiyan et al., 2006).

Inoculation of *Methylobacterium* significantly increased plant height and dry matter production in cotton than uninoculated control

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Table 1
Effect of PPFM isolates on plant height, tiller production and leaf area index of paddy at 60 days after transplanting.

Sl.No.	Isolate code No.	Plant height (cm)	Number of tillers hill ⁻¹	LAI
60 DAT				
1	PPFM1	62.85	32.67	4.49
2	PPFM2	67.55	34.67	4.48
3	PPFM3	68.80	34.67	4.36
4	PPFM4	66.90	39.00	4.47
5	PPFM5	65.55	32.00	4.38
6	PPFM6	67.40	35.67	4.66
7	PPFM7	65.30	38.67	4.64
8	PPFM8	67.40	24.00	3.81
9	PPFM9	63.25	25.00	3.95
10	PPFM10	64.95	29.00	3.89
11	PPFM11	63.10	25.67	3.90
12	PPFM12	68.00	29.67	4.49
13	PPFM13	70.40	31.67	4.52
14	PPFM14	68.90	24.00	3.81
15	PPFM15	67.95	40.67	4.56
16	PPFM16	67.95	27.00	4.28
17	PPFM17	70.75	29.67	4.20
18	PPFM18	70.70	34.00	4.68
19	PPFM19	71.15	41.67	4.81
20	PPFM20	64.35	32.00	4.57
21	PPFM21	71.40	41.00	4.78
22	PPFM22	70.30	29.67	4.45
23	PPFM23	64.85	24.67	3.88
24	PPFM24	65.40	26.00	4.12
25	PPFM25	65.35	27.67	4.37
26	PPFM26	72.80	32.67	4.63
27	PPFM27	70.50	25.67	4.04
28	PPFM28	72.05	36.00	4.74
29	PPFM29	62.45	24.00	3.81
30	PPFM30	71.20	32.00	4.59
31	PPFM31	62.10	26.67	4.20
32	PPFM32	62.25	27.67	4.36
33	PPFM33	68.15	24.00	4.09
34	PPFM34	60.35	25.00	3.96
35	PPFM35	60.45	25.67	4.04
36	PPFM36	61.95	27.00	4.28
37	PPFM37	64.95	24.67	3.91
38	PPFM38	58.95	26.67	4.20
39	PPFM39	61.10	26.67	4.20
40	PPFM40	62.75	29.00	4.45
41	PPFM41	57.00	19.67	3.89
42	PPFM42	60.25	26.00	3.88
43	PPFM43	64.95	25.67	3.85
44	PPFM44	66.45	24.67	3.81
45	PPFM45	60.70	24.00	3.83
46	PPFM46	63.60	25.00	3.90
47	PPFM47 (Reference strain)	68.20	31.00	4.38
48	Control	60.10	24.00	3.80
	CD (0.05)	2.397	3.181	0.527
	SEm (±)	0.85	1.13	0.19

(Madhaiyan et al., 2005). Madhaiyan et al. (2006) reported the presence of ACC deaminase enzyme which reduces the level of ethylene, in *Methylobacterium fujisawaense*. Lower levels of ethylene promote the root elongation of canola seedlings under gnotobiotic conditions. Radhika et al. (2008) recorded highest maize cob yield in plants sprayed with PPFMs. Pattanashetti et al. (2012) conducted a pot culture experiment to study the effect of selected methyloprophs on growth and yield of *Coleus forskohlii*. Results suggested that plant height, chlorophyll content, shoot biomass, leaf area, stem girth and tuber yield increased due to PPFMs treatment.

Rice, the most important cereal crop in the world, belongs to the family Poaceae. It is the most widely consumed staple food for over half

of the world's population. There are several reports on the beneficial effects of PPFMs on paddy. They promote the growth of paddy in a number of ways. Madhaiyan et al. (2004) observed higher photosynthetic activity in rice cultivar Co-47 that received *Methylobacterium* and attributed the effect due to enhancement of chlorophyll concentration, maleic acid content and increased number of stomata. Several workers reported growth promotional ability of PPFMs in several crops including cotton (Madhaiyan et al., 2005), tomato (Thangamani and Sundaram, 2005), soybean, blackgram and sugarcane (Madhaiyan et al., 2005). Lee et al. (2006) observed the effect of three plant-growth promoting, N₂ fixing methyloprophic strains *Methylobacterium* spp. CBMB20, *Enterobacter* sp. CBMB30, *Burkholderia* spp. CBMB40, on the early growth of rice. These three methyloprophic strains significantly improved seed germination, seedling vigour index (SVI) and biomass of rice seedlings. Eight *Methylobacterium* isolates were tested for their effect on seed germination. The isolates, PPFM-SOY (isolated from soybean leaf) and GN (isolated from groundnut leaf) increased the germination percentage of heat-treated seeds of soybean, maize and paddy (Anitha, 2010).

Considering the importance of PPFM as plant growth promoting bacteria, an attempt was made to select efficient PPFM strains based on growth and yield of paddy and their morphological, biochemical and molecular characterization.

In the present investigation PPFM cultures were grown for 7 days and 1 per cent of culture (10⁵ cfu ml⁻¹) was sprayed with a hand sprayer at the rate of 25 ml plant⁻¹ on the leaves in the morning to have uniform wetting as described by Holland and Polacco (1994) at 15 and 30 days after transplanting (DAT). The present investigation conclusively proved that, PPFM inoculation in paddy significantly improved growth parameters like plant height, tiller production and leaf area index compared to uninoculated plants.

As part of the study conducted by Nysanth et al. (2018) in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, during 2015–2017, Pink Pigmented Facultative Methyloprophs (PPFMs) were isolated from the phyllosphere of paddy collected from different agro climatic conditions of Kerala by leaf imprint method using Ammonium Mineral Salt (AMS) agar media supplemented with 0.5% methanol and cycloheximide. In all, 46 isolates were obtained and tested their ability to produce IAA (Indole-3 acetic acid) and carotenoids pigment, antagonistic activity against different phytopathogens of paddy and effect on seed germination and seedling vigour of paddy under *in vitro* conditions. The product developed by Tamil Nadu Agricultural University was taken as the reference culture (PPFM47).

In the present investigation, a pot culture experiment was conducted to study the effect of PPFM isolates on growth and yield of paddy using variety Jyothi (Ptb-39) in completely randomized design using wetland soil. The PPFM bioinoculant was prepared by inoculating 72 h old log phase culture in AMS broth (Whittenburry et al. 1970). The flasks were kept in a temperature controlled shaker at 28 ± 2 °C for 7 days. The 15 days old healthy seedlings were selected for transplanting. The seedlings were uprooted from nursery bed and dipped in 2 percent solution (10⁵ cfu/ml) of PPFM culture for 30 min before transplanting. After root dip two seedlings were transplanted per earthen pot containing 10.5 kg wetland soil. The PPFM cultures were grown for 7 days and prepared 1 percent solution (10⁵ cfu/ml) and sprayed with a hand sprayer at the rate of 25 ml/plant on the leaves in the morning to have uniform wetting as described by Holland and Polacco (1994) at 15 and 30 days after transplanting (DAT).

At 60 DAT, it was found that inoculation of PPFM26 recorded significantly higher plant height (72.80 cm) followed by PPFM28 (72.05 cm), PPFM 21 (71.40 cm), PPFM30 (71.20 cm), PPFM19 (71.15 cm), PPFM17 (70.75 cm), PPFM 18 (70.70 cm) and PPFM27

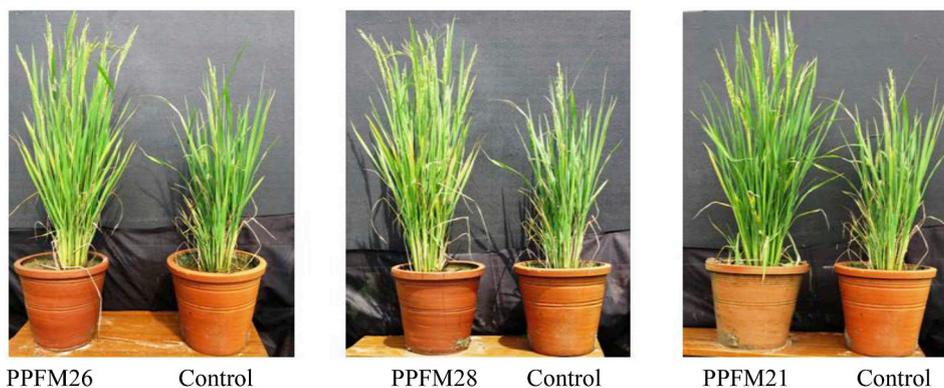


Fig. 1. Effect of PPFM isolates on height of paddy at 60 DAT.

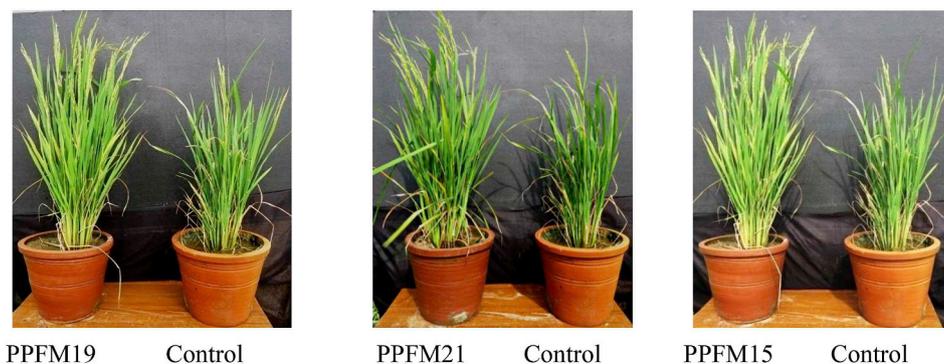


Fig. 2. Effect of PPFM isolates on tiller production of paddy at 60 days after transplanting.

(70.50 cm). Here PPFM26 was statistically on par with others. These treatments were found to be significantly superior to the control (60.10 cm) (Table 1) (Fig. 1).

The number of tillers hill⁻¹ was significantly influenced by inoculation of PPFM. At 60 DAT, plants inoculated with PPFM19 were found to be the best in tiller production (41.67) among all the treatments. This was statistically on par with other treatments PPFM21 (41.00), PPFM15 (40.67), PPFM4 (39.00) and PPFM7 (38.67). All these treatments were found to have significant effect on higher tiller production compared to control which recorded 24.00 tillers hill⁻¹ (Table 1) (Fig. 2).

Leaf Area Index was calculated at 50% flowering stage. Leaf Area Index was computed by the following formula developed by Watson (1947).

$$\text{Leaf Area Index} = \frac{\text{Leaf area plant}^{-1}(\text{cm}^2)}{\text{Land area occupied by the plant}(\text{cm}^2)} \times 100$$

Significant increase in Leaf Area Index (LAI) was observed in plants inoculated with PPFMs (Table 3). The treatment PPFM19 recorded the maximum LAI of 4.81 and this was statistically on par with other treatments such as PPFM21 (4.78), PPFM28 (4.74), PPFM18 (4.68), PPFM6 (4.66), PPFM7 (4.64), PPFM1 (4.49), PPFM2 (4.48), PPFM4 (4.47) and the reference strain PPFM47 (4.38) as against the control which recorded a LAI of 3.80 (Table 1).

Similarly, combined inoculation of PPFMs and *Rhizobium* on groundnut cultivar Co (Gn)4 gave significant increase in plant growth, biomass production and yield parameters of groundnut (Reddy, 2002). True seeds of sugarcane treated with PPFMs resulted in higher germination percent and rate of germination compared to the control. A combination of PPFM treatment (seed imbibitions, soil application and phyllosphere spray) increased plant height, specific leaf area, number

of internodes and cane yield (Madhaiyan et al., 2005). The results of the present study were in accordance with results of a two-year field experiment conducted by Gawad et al. (2015) which revealed that inoculation of PPFM alone achieved the highest significant increases in the number of leaves per plant, average leaf area, haulm fresh weight, leaf chlorophyll, pod number and yield per plant. In addition, it improved the quality of pods by increasing the amino acids, protein, total sugars and ascorbic acid content.

It was interesting to note that application of PPFM isolates significantly influenced the yield and yield attributes of paddy.

The number of panicles hill⁻¹ was significantly superior in plants inoculated with PPFM19 which recorded 33.00 panicles hill⁻¹ compared to the other treatments and the control which recorded a panicle number of 14.67 hill⁻¹ (Table 2). For panicle length, length of main axis of five randomly selected panicles of observational plants was measured from base to tip and the average expressed in 'cm'. Maximum panicle length of 24.25 cm was recorded in plants inoculated with PPFM16 (Table 2). However, this was statistically on par with other treatments such as PPFM10, PPFM11, PPFM9, PPFM6, PPFM25, PPFM17, PPFM1, PPFM13, PPFM3 and PPFM29 which recorded panicle length of 24.20, 24.05, 23.85, 23.75, 23.70, 23.65, 23.50, 23.45, 23.15 and 23.10 cm respectively (Table 2). These treatments were statistically superior to the control which recorded panicle length of 21.60 cm (Fig. 3). Number of grains panicle⁻¹ was significantly influenced by PPFM treatment (Table 2). Inoculation of plants with PPFM10 recorded significantly higher number of grains panicle⁻¹ (142.70) which was on par with treatments PPFM9 (136.00), PPFM11 (135.33), PPFM16 (134.00) and PPFM17 (130.80). All these treatments were found to be statistically superior to the control (99.33) (Table 2). The data indicated that filled grains panicle⁻¹ was significantly influenced by PPFM inoculation (Table 2). Treatment with PPFM11 recorded

Table 2
Effect of PPFM isolates on yield and yield attributes of paddy at harvest.

Sl. No.	Isolate code No.	Number of panicle hill ⁻¹	Panicle length* (cm)	Number of grains panicle ⁻¹ *	Filled grains Panicle ⁻¹ *	Sterility percentage*
1	PPFM1	24.67	23.50	108.83	103.33	5.05
2	PPFM2	27.67	21.60	124.50	116.67	6.43
3	PPFM3	25.00	23.15	125.50	113.67	9.54
4	PPFM4	21.00	22.00	100.00	92.67	7.59
5	PPFM5	28.00	22.10	109.70	99.00	9.99
6	PPFM6	19.67	23.75	108.67	101.00	6.91
7	PPFM7	28.67	22.70	121.33	112.33	7.88
8	PPFM8	22.67	22.90	120.30	112.00	6.58
9	PPFM9	26.00	23.85	136.00	122.67	9.99
10	PPFM10	25.00	24.20	142.70	127.33	10.85
11	PPFM11	22.67	24.05	135.33	131.00	3.31
12	PPFM12	20.00	21.95	115.00	108.67	5.73
13	PPFM13	21.00	23.45	110.17	103.00	6.27
14	PPFM14	19.67	21.85	101.83	93.33	8.75
15	PPFM15	27.67	22.95	104.20	98.33	5.74
16	PPFM16	24.00	24.25	134.00	127.67	4.87
17	PPFM17	22.67	23.65	130.80	124.00	4.98
18	PPFM18	25.00	22.90	124.00	115.67	6.85
19	PPFM19	33.00	22.50	111.80	102.67	4.08
20	PPFM20	23.67	22.95	126.20	121.33	3.95
21	PPFM21	24.00	21.90	108.00	98.00	9.24
22	PPFM22	23.67	21.95	118.70	111.33	6.27
23	PPFM23	20.67	21.80	100.37	88.67	11.43
24	PPFM24	24.00	22.20	83.00	68.67	17.48
25	PPFM25	19.67	23.70	87.50	83.67	4.41
26	PPFM26	23.67	22.35	104.20	97.00	6.97
27	PPFM27	26.00	22.05	124.33	120.33	3.12
28	PPFM28	30.00	22.25	99.83	88.33	11.37
29	PPFM29	21.67	23.10	115.17	108.00	6.08
30	PPFM30	16.67	22.80	106.50	102.00	4.25
31	PPFM31	21.67	22.30	117.17	110.67	5.55
32	PPFM32	19.00	22.30	101.30	75.67	8.15
33	PPFM33	18.67	22.10	108.70	106.33	2.31
34	PPFM34	28.67	21.60	107.67	101.00	5.99
35	PPFM35	24.67	21.25	110.50	103.67	6.33
36	PPFM36	16.00	22.05	110.00	99.67	9.56
37	PPFM37	23.67	23.15	112.00	106.00	5.29
38	PPFM38	19.67	21.90	117.67	111.00	5.50
39	PPFM39	15.00	21.60	117.30	113.00	3.41
40	PPFM40	20.67	18.90	102.83	72.33	13.49
41	PPFM41	15.00	22.40	99.60	96.67	10.32
42	PPFM42	18.00	22.70	106.33	98.00	7.99
43	PPFM43	22.00	21.80	107.50	102.67	4.78
44	PPFM44	22.00	21.80	114.50	109.00	4.76
45	PPFM45	23.67	22.50	90.70	83.33	8.22
46	PPFM46	26.67	22.25	115.70	102.33	11.71
47	PPFM47 (Reference strain)	23.67	21.85	100.20	89.33	11.00
48	Control	14.67	21.60	99.33	89.33	10.12
	CD (0.05)	2.524	1.175	15.701	16.781	4.192
	SEm (±)	0.90	0.42	5.58	5.97	1.49

* Mean of 5 replications.

significantly higher number of filled grains panicle⁻¹ (131.00). This was found to be statistically on par with other treatments such as PPFM16, PPFM10, PPFM17, PPFM9, PPFM20, PPFM27, PPFM2 and PPFM18 which recorded 127.67, 127.33, 124.00, 122.67, 121.33, 120.33, 116.67 and 115.67 respectively. These treatments were found to be statistically superior compared to control (89.33). The number of filled and unfilled grains panicle⁻¹ was obtained from ten randomly selected panicle separately and sterility percentage was worked out using the following formula:

$$\text{Sterility percentage} = \frac{\text{Number of unfilled grains per panicle}}{\text{Number of total grains per panicle}} \times 100$$

Results revealed that sterility percentage was significantly

influenced by PPFM treatments (Table 2). PPFM33 recorded the least sterility percentage of 2.31. This was on par with other treatments PPFM27 (3.12%), PPFM11 (3.31%), PPFM39 (3.41%) and PPFM30 (4.25%). These treatments were statistically superior to the control which recorded sterility percentage of 10.12 per cent.

The results indicated that thousand grain weight was significantly influenced by the PPFM treatments. Among all the treatments, PPFM16 recorded significantly higher thousand grain weight (30.66 g) which was on par with PPFM25 (28.97 g). These treatments were statistically superior to the control which recorded a thousand grain weight of 24.28 g. The thousand grain weight of the reference strain was 26.13 g (Table 3). Inoculation of PPFM significantly increased the yield of paddy. PPFM11 recorded the highest yield of 46.30 g hill⁻¹ and this

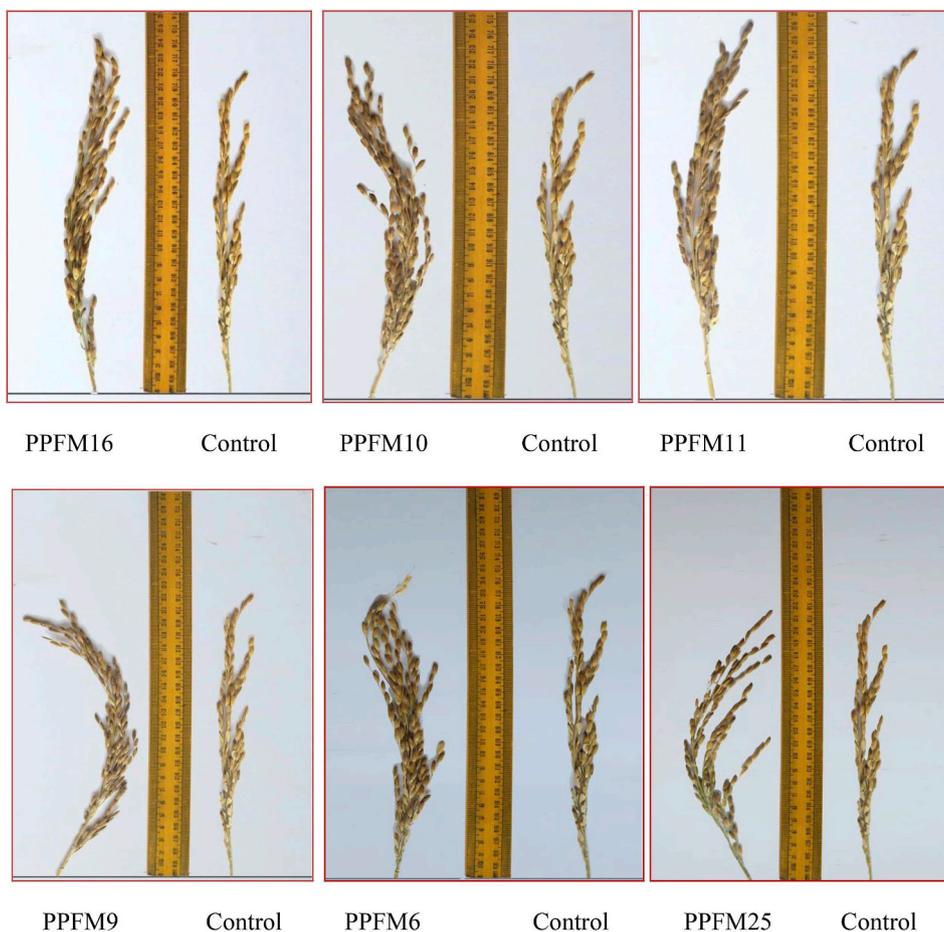


Fig. 3. Effect of PPFM isolates on panicle length of paddy at harvest.

was statistically on par with PPFM16 (45.05 g hill⁻¹), PPFM6 (44.75 g hill⁻¹), PPFM18 (43.95 g hill⁻¹), PPFM15 (43.25 g hill⁻¹), PPFM19 (42.95 g hill⁻¹), and PPFM9 (42.05 g hill⁻¹) whereas the control recorded a grain yield of 33.65 g hill⁻¹. Only 38.40 g hill⁻¹ of grain yield was obtained in plants treated with reference strain (Table 3).

The straw yield was significantly higher in plants treated with PPFM isolates. The isolate PPFM29 recorded the highest straw yield of 75.10 g hill⁻¹ followed by PPFM20 (73.50 g hill⁻¹), PPFM30 (71.60 g hill⁻¹), PPFM2 (71.50 g hill⁻¹) and PPFM21 (70.40 g hill⁻¹). The control plants recorded a straw yield of 51.40 g hill⁻¹ and the reference strain 65.00 g hill⁻¹ (Table 3). Analysis of the data on table 14 indicated that there was significant effect on dry matter production of paddy treated with PPFMs. Maximum dry matter production (136.70 g hill⁻¹) was recorded in plants inoculated with PPFM35, which were followed by PPFM37 (135.20 g hill⁻¹) and PPFM36 (133.80 g hill⁻¹) which were statistically on par, whereas, control recorded a dry matter production of 73.93 g hill⁻¹ (Table 3).

These results were in line with the study of Madhaiyan et al. (2006) who reported the presence of ACC deaminase, enzyme which reduces the level of ethylene, in *Methylobacterium fujisawaense*. Lower levels of ethylene promote the root elongation of canola seedlings under gnotobiotic conditions. In addition, Lee et al. (2006) observed the effect of three plant-growth promoting, N₂ fixing methylotrophic strains *Methylobacterium* spp. CBMB20, *Enterobacter* sp. CBMB30, *Burkholderia*

spp. CBMB40, on the early growth of rice. Moreover, Radhika et al. (2008) recorded highest maize cob yield in plants sprayed with PPFMs. Furthermore, Pattanashetti et al. (2012) conducted a pot culture experiment to study the effect of selected methylotrophs on growth and yield of *Coleus forskohlii*. Results suggested that plant height, chlorophyll content, shoot biomass, leaf area, stem girth and tuber yield increased due to PPFMs treatment. Among the various isolates tested PPFM50 was selected as the best. Inoculation of this isolate increased the tuber yield to 216.10% against uninoculated control and 136.07% against reference strain.

Chlorophyll content was estimated by DMSO (Dimethyl sulphoxide) method. The chlorophyll content of the plants significantly differed due to various inoculation treatments. At 60 DAT, the maximum chlorophyll content of 1.97 µg sample tissue⁻¹ was recorded in PPFM46 and PPFM18 and these were statistically on par with treatments such as PPFM12 (1.90 µg g⁻¹), PPFM11 (1.85 µg g⁻¹), PPFM16 (1.85 µg g⁻¹), PPFM42 (1.84 µg g⁻¹), PPFM2 (1.82 µg g⁻¹), the reference strain PPFM47 (1.80 µg g⁻¹), PPFM26 (1.79 µg g⁻¹), PPFM35 (1.77 µg g⁻¹), PPFM45 (1.77 µg g⁻¹), PPFM33 (1.75 µg g⁻¹), PPFM1 (1.73 µg g⁻¹), PPFM41 (1.73 µg g⁻¹) and PPFM8 (1.72 µg g⁻¹). All these treatments were found to be significantly superior to the control which recorded a total chlorophyll content of 1.40 µg g⁻¹ of sample tissue (Table 4).

Similar findings were already reported in many of previous studies. PPFM inoculation was found to increase the photosynthetic activity by

Table 3
Effect of PPFM isolates on yield and yield attributes of paddy at harvest.

Sl.No.	Isolate code No.	1000 grain weight (g)	Grain yield hill ⁻¹ (g)	Straw yield hill ⁻¹ (g)	Dry matter production (g/hill)
1	PPFM1	24.37	35.10	61.20	86.60
2	PPFM2	27.33	38.05	71.50	97.00
3	PPFM3	26.06	39.95	65.50	91.80
4	PPFM4	26.83	38.10	52.90	89.30
5	PPFM5	26.41	34.55	57.00	99.40
6	PPFM6	27.15	44.75	61.70	91.80
7	PPFM7	26.58	41.85	52.10	93.80
8	PPFM8	23.85	35.65	54.40	74.40
9	PPFM9	26.10	42.05	54.50	74.90
10	PPFM10	26.00	40.15	56.90	87.70
11	PPFM11	26.28	46.30	58.50	89.90
12	PPFM12	25.05	40.40	64.50	99.70
13	PPFM13	24.31	35.05	53.50	85.20
14	PPFM14	27.74	40.85	53.50	94.40
15	PPFM15	25.13	43.25	66.80	74.70
16	PPFM16	30.66	45.05	58.50	88.10
17	PPFM17	27.92	41.05	53.90	74.40
18	PPFM18	27.52	43.95	70.10	128.00
19	PPFM19	28.44	42.95	64.00	101.20
20	PPFM20	25.82	38.30	73.50	133.00
21	PPFM21	26.34	36.25	70.40	111.10
22	PPFM22	25.86	38.40	68.80	126.50
23	PPFM23	27.85	35.65	52.30	89.60
24	PPFM24	28.33	38.20	57.80	108.00
25	PPFM25	28.97	33.85	54.40	83.50
26	PPFM26	26.48	38.20	63.00	106.30
27	PPFM27	28.69	40.60	60.70	99.30
28	PPFM28	24.26	33.85	53.90	80.50
29	PPFM29	26.51	33.60	75.10	84.80
30	PPFM30	26.97	34.60	71.60	129.10
31	PPFM31	27.82	30.30	52.60	78.30
32	PPFM32	26.59	39.45	59.30	111.20
33	PPFM33	24.97	35.65	54.70	88.80
34	PPFM34	27.28	36.05	45.20	85.70
35	PPFM35	26.89	33.70	67.00	136.70
36	PPFM36	28.71	34.55	66.20	133.80
37	PPFM37	25.95	37.85	62.70	135.20
38	PPFM38	24.22	35.80	53.50	131.30
39	PPFM39	26.44	33.60	51.60	81.50
40	PPFM40	25.27	35.15	60.20	81.93
41	PPFM41	24.77	29.55	44.20	78.70
42	PPFM42	24.86	33.80	54.70	101.60
43	PPFM43	24.48	34.15	66.20	131.90
44	PPFM44	25.08	35.10	51.50	85.00
45	PPFM45	26.33	33.85	60.50	82.30
46	PPFM46	24.35	31.15	55.70	87.90
47	PPFM47	26.13	38.40	65.00	119.30
	(Reference strain)				
48	Control	24.28	33.65	51.40	73.93
	CD (0.05)	1.749	4.245	4.754	3.645
	SEm (±)	0.62	1.51	1.69	1.30

enhancing the number of stomata, chlorophyll concentration and malic acid content of crops (Martinus et al. 2004). Madhaiyan et al. (2004) observed higher photosynthetic activity in rice cultivar Co-47 that received *Methylobacterium* and attributed the effect due to enhancement of chlorophyll concentration, maleic acid content and increased number of stomata.

Proline content was estimated as per the procedure described by Bates et al. (1973). Inoculation of PPFM isolates showed significant increase in proline content over the control. The details are presented in Table 4. Proline content of the plants inoculated with PPFM ranged

Table 4
Effect of PPFM isolates on chlorophyll content, proline content and cell membrane stability index of paddy content at 60 DAT.

Sl.No.	Isolate code No.	Total Chlorophyll (mg g tissue ⁻¹)	Proline content (μ moles g tissue ⁻¹)	Cell membrane stability index (%)
1	PPFM1	1.73	30.61	77.90
2	PPFM2	1.82	149.84	72.30
3	PPFM3	1.43	11.20	64.10
4	PPFM4	1.71	30.24	72.23
5	PPFM5	1.65	59.86	75.97
6	PPFM6	1.62	82.30	74.77
7	PPFM7	1.46	21.86	66.83
8	PPFM8	1.72	78.93	74.23
9	PPFM9	1.45	164.81	77.30
10	PPFM10	1.68	285.51	71.50
11	PPFM11	1.85	248.39	66.80
12	PPFM12	1.90	40.16	73.13
13	PPFM13	1.59	118.43	64.80
14	PPFM14	1.62	224.89	78.97
15	PPFM15	1.63	261.39	68.07
16	PPFM16	1.85	270.76	71.37
17	PPFM17	1.59	8.09	68.23
18	PPFM18	1.97	29.98	66.20
19	PPFM19	1.72	285.04	63.17
20	PPFM20	1.49	5.09	64.17
21	PPFM21	1.45	11.24	67.90
22	PPFM22	1.46	175.32	73.53
23	PPFM23	1.42	24.78	72.40
24	PPFM24	1.41	167.60	69.83
25	PPFM25	1.42	23.98	72.70
26	PPFM26	1.79	77.50	64.80
27	PPFM27	1.69	187.84	76.97
28	PPFM28	1.44	263.51	80.83
29	PPFM29	1.58	200.54	79.70
30	PPFM30	1.41	58.21	78.87
31	PPFM31	1.66	33.42	79.70
32	PPFM32	1.70	99.58	80.70
33	PPFM33	1.75	51.51	79.40
34	PPFM34	1.54	123.89	69.16 7
35	PPFM35	1.77	29.14	75.10
36	PPFM36	1.49	38.44	80.10
37	PPFM37	1.59	104.19	79.43
38	PPFM38	1.71	68.09	78.13
39	PPFM39	1.49	39.52	79.33
40	PPFM40	1.58	55.04	78.43
41	PPFM41	1.73	55.52	79.53
42	PPFM42	1.84	36.87	77.80
43	PPFM43	1.63	62.86	80.67
44	PPFM44	1.68	56.95	79.06
45	PPFM45	1.77	13.01	79.10
46	PPFM46	1.97	30.55	79.36
47	PPFM47	1.80	139.10	79.10
	(Reference strain)			
48	Control	1.40	36.79	
	CD (0.05)	0.258	12.832	3.286
	SEm (±)	0.09	4.56	1.17

between 5.09 and 285.51 μ moles g tissue⁻¹. Plants inoculated with PPFM10 recorded the maximum value of 285.51 μmol g tissue⁻¹, which was on par with proline content (285.04 μmol g tissue⁻¹) of plants inoculated with PPFM19. These treatments were significantly superior to control which recorded a proline content of 36.79 μmol g tissue⁻¹.

Proline is one of the most important osmolytes that accumulate in plants during severe drought stress (Yoshida et al., 1997). It not only acts as an osmolyte for osmotic adjustment but also helps to stabilize sub-cellular structures (e.g. proteins and membranes). It is also involved

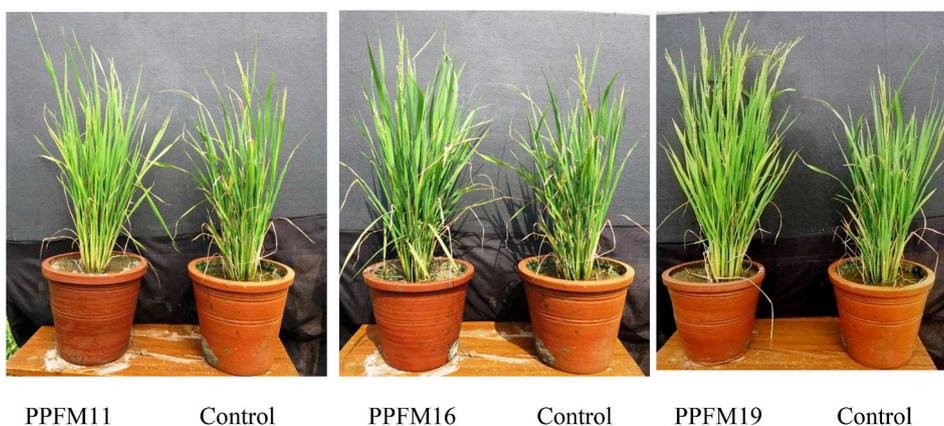


Fig. 4. Plants treated with selected PPFM isolates at 60 days after transplanting.

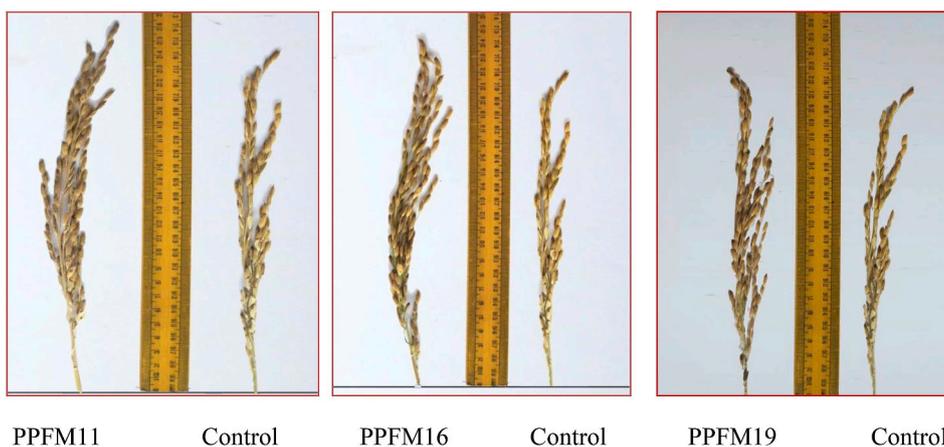


Fig. 5. Effect of selected PPFM isolates on panicle length of paddy at harvest.

Table 5
Morphological characterization of selected PPFM isolates.

Sl. No.	Isolate code No.	Cell shape	Motility	Gram reaction	Pigmentation
1	PPFM11	Rod	Positive	Negative	Dark Pink
2	PPFM16	Rod	Positive	Negative	Pale pink
3	PPFM19	Rod	Positive	Negative	Pale pink

in the scavange of free radicals and buffering cellular redox potential. The results of the present study was also in agreement with the study of Sivakumar et al. (2017) who reported treatment of plants with *Methylobacterium* spp. has to lead to an increase in proline levels. Foliar application of PPFM (2%) increased the proline content by 11.34 per cent in tomato compared to absolute control under stressed condition. Here the treated plant recorded $326.45 \mu\text{g g}^{-1}$ of proline as against $162.66 \mu\text{g g}^{-1}$ in absolute control.

Cell membrane stability was estimated as per the procedure described by Blum and Ebercon (1981). The data on the effect of inoculation of PPFM isolates on plant cell membrane stability index are presented in Table 4. Data showed that PPFM treatments had significant influence on plant cell membrane stability. A significantly higher cell membrane stability index of 80.83% was recorded with PPFM28, which was statistically on par with PPFM32, PPFM43, PPFM36, PPFM29,

PPFM31, PPFM41, PPFM37, PPFM33, PPFM46, PPFM39, PPFM45, PPFM47 (reference strain), PPFM44, PPFM14, PPFM30, PPFM40, PPFM38, PPFM1 and PPFM42 which recorded cell membrane stability index of 80.70, 80.67, 80.10, 79.70, 79.70, 79.53, 79.43, 79.40, 79.36, 79.33, 79.10, 79.10, 79.06, 78.97, 78.87, 78.43, 78.13, 77.90 and 77.80 per cent respectively.

These results confirmed previous studies with increase of rice and sugarcane growth, when these crops were treated with *Methylobacterium* strains (Madhaiyan et al., 2005).

The isolates PPFM11, PPFM16 and PPF19 were adjudged as superior isolates based on growth promotion efficacy and grain yield and yield attributes of paddy (Figs. 4 and 5). These three isolates were identified as *Methylobacterium* spp. based on morphological, biochemical and molecular characteristics. The superior isolates selected were subjected to morphological characterization and the results are presented in Table 5. The results revealed that all the isolates were rod shaped, stained gram negative and exhibited motility. Further one week after incubation, pink colonies with different color intensities were observed on AMS medium. Out of three efficient isolates, two isolates exhibited pale pink colored colonies, whereas one isolate was found to be of dark pink color. The colony morphology of these isolates is presented in Fig. 6.

The results of the present study was in agreement with the

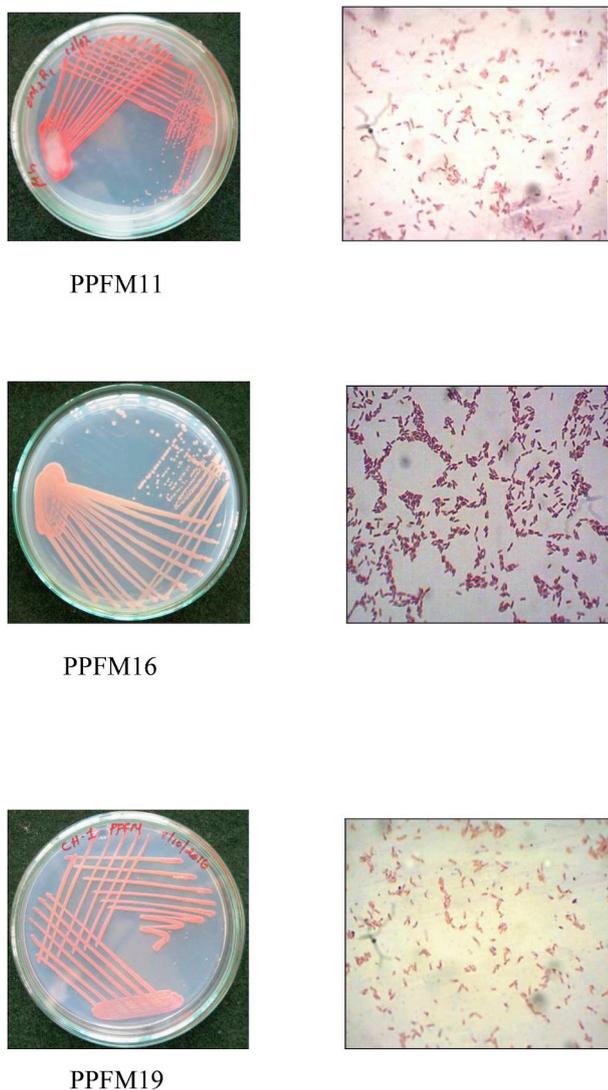


Fig. 6. Colony morphology of the selected PPFM isolates and Gram reaction.

microscopic studies of Green and Bousifield (1982) which revealed that all the PPFM isolates were rod shaped, motile, gram negative and produced poly β -hydroxy butyrate granules. In the present study, the superior isolates selected were subjected to morphological characterization. The results revealed that all the isolates were rod shaped, stained Gram negative and exhibited motility. Further one week after incubation, pink colonies with varying color intensities were observed on AMS medium. The expression of pink pigmentation with varied level of intensity in PPFM indicates the presence of carotenoids (Fasim, 2003) which is known to protect these bacteria from intense light and UV radiation (Liu et al., 1993).

For further characterization, these three selected isolates were subjected to a series of biochemical tests. Biochemical characterization of selected bacterial isolates was done by performing various biochemical tests and carbohydrate utilization tests by using readymade Himedia[®] kits (HiCarbo™, Part A, Band C, Hi25™ Enterobacteriaceae). Color change observed on the biochemical amended media of the kit after spot inoculating culture suspensions of selected isolates followed

Table 6
Utilization of different carbon substrates by selected PPFM isolates.

Sl. No	Carbon substrate	PPFM11	PPFM16	PPFM19
1	D- Glucose	+	+	+
2	D- Fucose	-	+	-
3	D- Xylose	-	+	-
4	L- Arabinose	+	+	-
5	D- Fructose	+	-	+
6	L- Aspartate/L- Glutamate	-	+	-
7	Sebacate	-	-	-
8	Acetate	+	+	+
9	Betaine	+	-	+
10	Tartarate	-	-	+
11	Ethanol	+	+	+
12	Methylamine	-	-	+
13	Dimethylamine	+	-	+
14	Formaldehyde	+	+	+
15	Glycerol	+	+	+
16	Methanol	+	+	+
17	Formate	-	+	+
18	Succinate	-	+	+
19	Lactate	+	+	+
20	Pyruvate	+	+	+
21	Salicylate	-	-	-
22	Nutrient agar	+	+	+
23	Fumarate	+	+	+
24	Rhamnose	-	-	-
25	Raffinose	-	-	-
26	Esculine	-	-	-
27	Cellobiose	-	-	-
28	Melibiose	-	-	-
29	Saccharose	-	-	-

by incubation for 72 h indicated the reaction with respect to different biochemicals or carbohydrates as positive or negative. Using the results of various biochemical tests, a tentative genus level identification was done. All the 3 isolates were identified to be belonging to genus *Methylobacterium*. All the isolates confirmed to be negative for Methyl red, Voges-Proskauer test, Phenylalanine deamination, H₂S production, Arginine lyase utilization and positive for Oxidase, Urease, Catalase activity, Indole production, Citrate, Lysine and Malonate utilization. None of the isolates could reduce nitrate to nitrite. All the selected isolates were tested for the utilization of 29 different carbon compounds and they showed wide variability in carbon utilization pattern (Table 6).

In support of these findings Lidstrom (1992) reported that methylotrophic bacteria are capable of growing on single carbon compounds such as formate, formaldehyde and methanol as sole source of carbon and energy. They can utilize wide range of multi carbon growth substrates making them facultatively methylotrophic. Based on their carbon utilization pattern, classification of methylotrophic bacteria at the species level has been established by Green and Bousifield (1982). All isolates were aerobes producing catalase and oxidase as already demonstrated by Bellin and Spain (1976) and positive for urease test and indole production (Thangamani and Sundaram, 2005). However, hydrolysis of casein, starch, cellulose degradation, MR and VP test and nitrate reduction test was not recorded in any of the isolates.

The present study demonstrated that it is possible to distinguish and classify the methylotrophic bacteria using 16 S rRNA sequence analysis. Our results also indicated that phylogenetic relationships based on 16 S rRNA sequences reflect the classical taxonomic classification systems based on phenotypic characteristics for methylotrophs. Thus, 16 S rRNA sequence analysis could be a useful tool for detailed classification of

Table 7
BLAST search details of the sequences producing most significant alignment of the bacterial isolates.

Isolate	Description	Max score	Total score	Query cover (%)	E value	Identity (%)	Accession no.
PPFM 11	<i>Methyllobacterium aquaticum</i> DNA, complete genome, strain: MA-22 A	2145	21221	100	0.0	99	AP 014704.1
PPFM 16	<i>Methyllobacterium radiotolerans</i> strain VR16-3 16 S ribosomal RNA gene, partial sequence	1951	1951	100	0.0	97	KY 882067.1
PPFM 19	<i>Methyllobacterium populi</i> strain BJ001 16 S ribosomal RNA, partial sequence	2207	2207	100	0.0	99	NR 074257.1

methylotrophs. 16 S rRNA gene phylogenetic analysis performed clearly showed the position of the isolates within the genus *Methyllobacterium*. Isolates PPFM11 and PPFM16 were found to be very close to *M. aquaticum* and *M. radiotolerans* respectively whereas PPFM19 was found to be very close to *M. populi* (Table 7).

In the present investigation, three PPFM isolates were selected as superior isolates. Further studies on the effect of these isolates on plants are required before developing commercial formulations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101055>.

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