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Development of Pink Pigmented Facultative Methylotrophs' (PPFMs) Consortium Formulation and Its Efficacy on Chilli (*Capsicum annuum*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This research aims to investigate the potential effect of Pink Pigmented Facultative Methylotrophs (PPFMs) as foliar application to enhance growth parameters of chiili crop. The laboratory and pot studies were conducted at Department of Agricultural Microbiology, Anand Agricultural University, Anand. The PPFMs were isolated from the phyllosphere of solanaceous crop (Chilli, Brinjal, Potato, Tomato and Tobacco) and five out of thirty isolates were selected based on their methanol consuming and plant growth promoting traits. Consortium of isolates (*Methylobacterium populi* AAU PPFM C-7, *M. radiotolerans* AAU PPFM C-17, *M. populi* AAU PPFM C-19, *M. populi* AAU PPFM T-2 and *M. radiotolerans* B-2) were prepared after studying their compatibility. Pot study was laid out in net house using completely randomized design with four replications on Chilli crop. The results of the study showed significant effect of PPFMs consortium on growth parameters of chilli (*viz.* shoot length, root length, shoot weight, root weight, number of branches per plant) that could be result of the plant growth promoting traits of PPFMs.

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1. INTRODUCTION

The plant canopy is a habitat to a wide range of microorganisms that have beneficial, harmful or neutralistic effects. Large fraction of the earth's surface is covered with plants, that leaf surfaces often represent a substantial multiple of the soil surface area. The terrestrial leaf surface area is over 6.4 × 10^8 km^2 that might be colonized by microbes to harbor bacterial population of about 10^{26} cells [1]. As an ecological niche, the plant phyllosphere supports highly abundant methylotrophic species ranging from 10^4 to 10^7 colony forming unit (CFU) per leaflet [2]. The phyllosphere represents the largest biological surface, estimated to be two times the surface of the earth and is involved in many processes required for the ecosystem including primary biomass production (food and feed), oxygen release and carbon-dioxide fixation [3]. Plant leaf surface is considered a hostile environment for bacterial epiphytes. Morphological feature of leaf surface and its surroundings both make phyllosphere ecosystem crucial than another habitat. Healthy leaf epidermal cells constantly secrete organic and inorganic molecules, such as amino acids, sugars (including sucrose, glucose and fructose), methanol and various salts to their surfaces [4,5].

Bacteria are the dominant inhabitants of crop phyllosphere and mainly represent on leaves in a large number. Pink Pigmented Facultative Methylotrophs (PPFMs) are heterotrophic microflora of the surface of young leaves and are group of Prokaryotic aerobic eubacteria. They are the endophytic prime inhabitants of wide variety of plant species [6]. The distinctive pink pigmentation is due to carotenoids render PPFM to be tolerant to extreme light condition and reactions, these features could explain their occurrence in diverse ecological system such as soil, plants, air and water [7]. Most common niche for synergism between *Methylobacterium* and plant is the phyllosphere, especially leaf surface. The genus *Methylobacterium* is one of the dominant genera, which act as a symbiont with plants by consuming methanol and in turn providing vitamin B_{12}, auxins and cytokinin useful for the plant growth and systemic resistance [8]. Their capacity for adapting to changing environmental conditions, growing at high rates on methanol and a variety of C1-Cn compounds (facultative) reflects their metabolic efficiency. Thus, broadly the *Methylobacterium* species are non-pathogenic and ubiquitous, found in variety of habitats on earth [9].

Chilli is an important vegetable and spice crop grown throughout the world. In India, it occupies the area of 287.05 thousand of hector with production of 3406.03 thousand tons. Foliar spraying for crop nutrition has gained the attention of researchers and farmers due to their superior effect. Plants are able to absorb essential elements through their leaves in foliar feeding. The absorption takes place through their stomata and also through their epidermis. Transport is usually faster through the stomata, but total absorption may be as great through the epidermis. Foliar feeding of PGPR and their metabolites has become standard practice now a day in smart agriculture for cultivation of vegetables and fruits and that's why we attempted study to role of native pink pigmented facultative methylotrophic (PPFMs) foliar inhabitant bacterial isolates as plant growth promoting probiotics was studied.

2. MATERIALS AND METHODS

2.1 Sources of Native PPFMs Bacterial Isolates

The PPFMs were isolated from the phyllosphere of solanaceous crops (*i.e.*, chilli, tomato, brinjal, tobacco and potato) from research farms of Anand Agricultural University (AAU) farms by leaf imprinting method [10].

2.2 Compatibility Test of PPFM Isolates

In vitro plate bioassay was carried out to determine compatibility of the native phyllospheric PPFM isolates. Each phyllospheric PPFM bacterial isolate was grown in NMS broth for 5-6 days. On Nutrient agar plate, one isolate (100 µl) was spreaded and the well was made by cork borer, in the well of the plate 50 µl of the other phyllospheric PPFM isolates were filled. The plates were incubated at 30+2˚C for 5 days and observed every 24 h for inhibition of the test cultures if any [11].

2.3 Consortia Preparation

PPFM bacterial isolates were grown in broth (Nutrient broth supplemented with 0.5% methanol) to ensure maximum number. Determination of population density of each isolate in broth was done by Neubauer hemocytometer. Individual culture in specific proportion was mixed to reach population density of 5 x 10^8 in final product and used for experiment.

2.4 Experimental Design

Pot experiment was conducted in net house during *kharif* season of the year 2017-18 at Department of Agricultural Microbiology, AAU, Anand. The details of the experiment described below.

Table 1. Treatment details

Treatment details	
T_1	Treated check: Chemical foliar fertigation (19:19:19) @ 2 g/lit
T_2	Indigenous Cow urine @ 2% v/v
T_3	Vermiwash @ 10% v/v
T_4	Phyllospheric PPFMs Consortium (5 × 10^8 CFU/ml)
T_5	AAU Methylotrophic bacterial consortium (phyllospheric and rhizospheric) for Rice (5× 10^8 CFU/ml)
T_6	Absolute control (Water spray)

2.5 treatment Application

The experiment was conducted in completely randomized design (CRD) with six treatments and four replications in Pot. The size of the pot was 10 kg soil/pot. The experiment was taken on Chilli crop (Variety- Gujarat Vegetable chilli – 111). All the treatments were given fertilizer as per the standard recommended fertilizer dose by AAU for that particular area (100-50-50 NPK kg/ha). Treatment as foliar spray at 30 and 45 days after transplanting (DATP) were given in T_1, T_2 and T_3 as mention in the Table 1. T_4 and T_5 were sprayed at 30 and 45 DATP @ 1 l/ha (5 × 10^8 CFU/ml).

2.6 Effect of PPFM Consortium on Growth Attributing Characters

Shoot and root length:

Shoot (from collar to tip of the plant) and root (collar to tip of root end) length was measured in centimeters with the help of 100 cm scale.

Chlorophyll content:

Chlorophyll content of the leaves was measured using atLEAF chlorophyll meter.

Phyllosphere PPFMs population:

The microbial population was enumerated on AMS media supplemented with methanol 0.5%.

No. of branches:

No. of branches were counted manually

Root & weight:

Fresh weight of root and shoot separately were weighed (g/plant) and recorded for the plants.

2.7 Statistical Analysis

Collected observations were analyzed for CRD design using GW-BASIC programme and interpretation was drawn from it.

3. RESULTS AND DISCUSSION

The phyllospheric isolates were screened based on their growth on AMS media supplemented with methanol as sole carbon source, methane monooxygenase, methanol dehydrogenase, IAA, ACC deaminase, Siderophore production, Lipase, Protease enzymes as plant growth promoting activities. From 30, five potent PPFMs isolates C-7, C-17, C-19, T-2 and B-2 were selected for consortium preparation and to study its efficacy on chilli crop in pot study.

Table 2. Effect of PPFMs consortium on shoot length

Treatments	30 DATP	45 DATP	90 DATP
T_1	22.45	36.00^b	70.25^e
T_2	22.08	36.65^b	76.25^d
T_3	21.50	36.57^b	79.33^c
T_4	22.58	38.93^a	88.42^a
T_5	22.43	36.86^b	83.17^b
T_6	22.51	35.44^b	66.50^f
C.V. %	3.30	2.73	1.85
S.Em. ±	0.37	0.50	0.72

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance

Table 3. Effect of PPFMs consortium on chlorophyll content (mg/cm²)

Treatments	30 DATP	45 DATP	90 DATP
T_1	46.73	40.48^{b}	51.17^{cd}
T_2	45.37	41.36 ^{ab}	52.08^{bcd}
T_3	45.53	41.76 ^{ab}	53.46^{b}
T_4	45.99	42.82^{a}	57.26^{a}
T_5	45.65	41.43^{ab}	53.23^{bc}
T_6	45.53	38.38^{c}	50.64^{d}
S.Em. ±	0.33	0.70	0.72
C.V. %	1.42	3.43	2.70

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance

Shoot length:

The observation on shoot length of the plant were measured at 30, 45 and 90 DATP and analyzed that showed no significant difference in plant height at 30 DATP, but at 45 DATP and 90 DATP it showed significant effect as T_4 was had the highest shoot length (38.93 cm) which was found significantly superior over T_6 (35.44 cm). Similarly at 90 DATP T_4 showed the highest shoot length (88.42 cm) which was found significantly superior over T_6 showing (66.50 cm), Which was followed by T_5 (83.17 cm), T_3 (79.33 cm), T_2 (76.25 cm) and T_1 (70.25 cm). T_4 was statistically superior over T_1. The increased shoot length might be the result of IAA production as it has important role in stimulation of cell elongation and division that aids to longer and PPFMs have been reported to produce IAA, Similar findings were reported by Subhaswaraj et al. [6] as they studied the impact of seed bacterization with *M. extorquens* MM2 on seed germination, root and shoot length where they found that *M. extorquens* MM2 has capacity to produce IAA (6.16 µg/ml) which resulted in increased seed vigour index and its foliar application contributed a substantial impact to growth of tomato plant. Senthilkumar and Krishnamoorthy [12] also reported that application of *Methylobacterium* by 1% foliar spray resulted in significant increase in plant height compared to control.

Effect of PPFMs consortium on chlorophyll content of Chilli *cv.* GVC-111:

The observation on chlorophyll content of leaves were measured at 30, 45 and 90 DATP that showed no significant difference at 30 DATP but at 45 and 90 DATP, the difference were significant. At 45 DATP, T_4 (42.82 mg/cm²) was found significantly superior over T_6 (38.38 mg/cm²) and T_5 (41. 43 mg/cm²), T_3 (41.76 mg/cm²) and T_2 (41.36 mg/cm²) found at par with the T_4. Similarly at 90 DATP T_4 showed the highest chlorophyll content (57.26 mg/cm²) which was found significantly superior over T_6 (50.64 mg/cm²), which was followed by T_3 (53.46 mg/cm²) and T_5 (53.23 mg/cm²). The siderophore production by the PPFMs may have contributed to the increase in chlorophyll content as iron is a crucial component of chlorophyll molecules, so improved iron uptake leads to higher chlorophyll synthesis. PPFMs also interact with plant hormones like auxins and cytokinins. These hormones can stimulate chlorophyll biosynthesis by upregulating the expression of relevant genes. Many other workers have also reported the effect of PPFMs on chlorophyll content. *Methylobacterium* application has showed increased in photosynthetic activity by increasing the stomatal count, chlorophyll content and malic acid content in crops [13]. Awasthy et al. [14] reported that 2% foliar spray of PPFM at 30 and 50 days increased chlorophyll content in Paddy. Satyan et al. (2018) reported increase the chlorophyll stability index of small cardamom due to PPFM inoculation. Our study also has a similar trend as evidenced by the higher chlorophyll content in the treatments compared to the controls.

Effect of PPFMs consortium on phyllosphere microbial population of chilli *cv.* GVC-111:

The PPFMs bacterial population were measured on 30, 45 and 90 DATP at initial and three days after application of respective treatment. Results of the same showed no significant difference 30 DATP initial, but the observation after three days of treatment application revealed that T_4 was having highest PPFMs population (5.52 log cfu/g of leaves) which was found significantly higher and T_5 (5.47 log cfu/g) was at par with T_4. Similarly at 45 DATP T_4 was having the significantly higher PPFMs population both at initial (4.51 log cfu/g) and three days after treatment application (5.50 log cfu/g) which were at par with T_5 initially (4.49 log cfu/g) and three days after treatment application (5.49 log cfu/g). At 90 DATP the PPFMs population data showed that T_4 having was significantly higher (5.52 log cfu/g) population which was at par with T_5 having (5.48 log cfu/g). Higher bacterial populations in of T_4 (PPFMs consortium) and T_5 (Rhizospheric and phyllospheric methylotrophs) after foliar spray shows their survival and proliferation in phyllosphere which is believed as their habitat.

Table 4. Effect of PPFMs consortium on bacterial population

Treatment	Phyllosphere bacterial population (log CFU/g of leaves)				
	30 DATP		45 DATP		90 DATP
	Initial	After 3 Days of spraying	Initial	After 3 Days of spraying	
T_1	4.41	4.42^b	4.39^b	4.39^b	4.39^b
T_2	4.40	4.40^b	4.37^b	4.38^b	4.38^b
T_3	4.39	4.41^b	4.37^b	4.38^b	4.41^b
T_4	4.40	5.52^a	4.51^a	5.50^a	5.52^a
T_5	4.41	5.47^a	4.49^a	5.49^a	5.48^a
T_6	4.38	4.39^b	4.36^b	4.37^b	4.36^b
S.Em. ±	0.04	0.03	0.03	0.04	0.04
C.V. %	1.68	1.43	1.36	1.52	1.47

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance
Logarithmic transformation given to microbial population values

Effect of PPFMs consortium on root weight, shoot weight, root length, shoot length, number of branches and yield of chilli *cv.* GVC-111:

Table 5. Effect of PPFMs consortium on root weight, shoot weight, root length, shoot length and number of branches chilli *cv.* GVC-111

Treatment	Root weight (g/plant)	Shoot weight (g/plant)	Root length (cm)	No. of Branch per plant
T_1	5.37^d	70.25^d	18.85^c	1.72^a
T_2	6.08^c	77.65^c	19.95^{bc}	1.75^a
T_3	6.79^b	80.05^{bc}	20.51^b	1.85^a
T_4	8.26^a	91.87^a	28.29^a	1.88^a
T_5	7.92^a	80.78^b	21.12^b	1.78^a
T_6	5.08^d	65.17^e	15.16^d	1.44^b
S.Em. ±	0.24	0.88	0.42	0.06
C.V. %	7.17	2.27	6.82	6.63

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance

Fig. 1. Chilli plant

Fig 2. Root of Chilli plant

Square root transformation was given to No. of branches values:

The observation on root weight, shoot weight, root length, shoot length and no. of branches were recorded and analyzed which shows that root weight of T4 (8.26 g/plant) was significantly superior than T_6 (5.08 g) and T_5 (7.92 g) found at par with T_4. Similarly, T_4 was having root length (28.29 cm) which was significantly higher than T_6 (15.16 cm). The observation on shoot weight also found significant, where T_4 (91.87 g/plant) was significantly superior than T_6 (65.17 g/plant). No. of branches were also significantly higher in T_4 (1.88) compared to T_6 (1.44). Nalayini et al. [15] also reported that application of 75% recommended N and P fertilizers with azophosmet and foliar application of PPFMB either 45 or at 60 DAS resulted in 2.3 q of additional seed cotton than 100% RDF without bioinoculants. Shivakumar et al. [16] reported that foliar application of PPFM has led to increase in root length and root volume on tomato crop under pot experiment. The enhancement PPFM (2%) might be due to involvement in the translocation of photo assimilates from source to sink. Similar results for growth parameters were reported in our study for growth attributing parameters.

4. CONCLUSION

In this study, foliar application of PPFMs consortium has resulted in enhanced growth parameters significantly in chilli under pot house experiment. The shoot length, chlorophyll content, root length, root weight, shoot weight and no. of branches per plant were found significantly higher in the treatment receiving PPFMs consortium compared to control. The Plant growth promoting regulators (IAA, ACC deaminase, siderophore production) has upregulated the plant growth which reflected in overall growth.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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