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Production of Plant Growth Hormones by Pink Pigmented Facultative Methylotrophs

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Methylotrophic bacteria like pink pigmented facultative methylotrophs (PPFMs) which are present on leaf surfaces take up methanol from the wastes of plant metabolism and contribute to growth and development of host plant in a number of ways. Using the leaf imprint method [1] and serial dilution technique, as many as 200 PPFM isolates were obtained from chilli leaves, rhizosphere soil and roots samples collected from 76 different locations and were tested for the production of plant growth promoting substances (IAA and GA). The production of IAA and GA varied among different PPFM isolates from 19.77 µg/ml to 0.14 µg/ml of culture filtrate and 128.28 µg/ ml to 4.77 µg /ml of culture filtrate respectively.

Key words: PPFM, Indole acetic acid, Gibberellic acid, HPLC.

Methylotrophic bacteria utilize single carbon (C_1) compounds for energy and carbon assimilation and are an important component of the global carbon cycle[2,3]. One group of methylotrophs, pink-pigmented facultative methylotrophic bacteria (PPFMs), is distinguished based on their formation of pink to red colonies on selective isolation media. PPFMs are a physiologically interesting group of bacteria that are capable of growing on single carbon such as methanol and methylamine, as well as on C_2, C_3, and C_4 compounds[4]. PPFM were first isolated as covert contaminants from the tissue cultures of liverwort, *Scapania nemorosa*[5], but later identified as belonging to the genus *Methylobacterium*. PPFMs are associated with the roots, leaves and seeds of most terrestrial plants. These bacteria are phytosymbionts that consume waste products such as methanol produced by the plants[6] and synthesize a variety of metabolites useful for the plants including phytohormones[7,8] that promote plant growth and yield. There are reports of production of the cytokinin zeatin[9] and indole acetic acid[7] by PPFMs.

The presence of indole-3-acetic acid (IAA) in supernatants of pink-pigmented facultative methylotrophic (PPFMs) bacterial cultures was observed[10]. Three out of the 16 isolates tested showed a positive reaction in a colorimetric assay. The presence was further unambiguously confirmed by high-performance liquid chromatography in combination with NMR. Gibberellic acid production by *Methylobacterium* sp and the amount of GA production was found to vary with strains ranging from 10. 9 mg to 106. 97 mg/ml of the culture broth[11].

In this point of view, an attempt was made to explore the diversity of PPFMs on major vegetable crop, chilli by different isolation techniques and also estimation of phytohormones

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like IAA and GA production by PPFMs was carried out at Department of Agricultural Microbiology, U A S Dharwad, Karnataka state, India.

MATERIALS AND METHODS

Collection of samples

Samples of leaves, roots and rhizosphere soils were collected from chilli (*Capsicum annum*) plants in fifteen villages each of three districts (Dharwad, Gadag and Haveri) of North Karnataka.

Isolation of PPFMs from *Capsicum anuum*

PPFMs were isolated on ammonium mineral salts medium (AMS). After sterilization of the media methanol (0.5%) was added as a sole carbon source and filter sterilized cyclohexamide (30 µg /ml) antibiotic was added.

Leaf imprinting technique [1]

On the solidified AMS agar medium upper and lower surface of leaf samples were placed separately, in such a way to make leaf imprint and incubated at 30^0 C for 5 days.

Serial dilution technique

One gram sample of rhizosphere soil and root was ground separately using a pestle and mortar, serially diluted upto 10^{-6} while 10^{-4}, 10^{-5} and 10^{-6} dilutions were used for isolation. Characteristic pink colonies growing over the medium were identified[13]. Further, the methylotrophs were purified by the streak plate method and well isolated colonies on the plates were preserved on AMS agar slants at 4°C for further use.

Morphological Characterization of PPFMs

The isolates were examined for their cell shape, motility studies, Gram reaction and pigmentation of colonies.

Production of phytohormones by PPFMs

Indole acetic acid production

Extraction

After seven days of incubation in AMS broth, 25 ml of the sample was withdrawn and the culture was centrifuged at 12,000 rpm for 30 min and the cell free culture filtrate was extracted twice with equal amount of ethyl acetate[14].

High performance liquid chromatography (HPLC) analysis of IAA

The ethyl acetate fractions of the cell free culture broth of PPFM isolates were used for Indole-3-acetic acid quantification by HPLC. The ethyl acetate was evaporated under vacuum, the sediment redissolved in 2 ml of absolute methanol, membrane filtered and 10 µl quantity was injected into the HPLC. Retention times for peaks were compared to those of authentic standards. Quantification of Indole-3-acetic acid compounds in the sample was done by comparison of retention time with peak height of chemical grade IAA (Sigma grade). Substances were quantified by integrating the areas under the peaks using UV detector at 254 nm[14, 10]

Gibberellic acid (GA) production

Extraction of gibberellins

Four days old PPFM culture was centrifuged for 15 min at 10,000 rpm and the supernatant was taken. The cell pellet was re-extracted with phosphate buffer (pH 8.0) and again centrifuged. Both supernatants were pooled, acidified at pH 2.5 using 5 N hydrochloric acid and partitioned with equal volumes of ethyl acetate for five times. The ethyl acetate phase was dried at 32°C and the residue redissolved in 2 ml of distilled water[14].

Estimation of gibberellins by spectrophotometry (GA)

Fifteen ml of ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added. After 2 min, 2 ml of potassium ferrocyanide solution was added and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 per cent hydrochloric acid and the mixture was incubated at 20°C for 75 minutes. The blank was prepared with 50 per cent hydrochloric acid. The absorbance was measured at 254 nm in a spectrophotometer. From the standard graph using standard gibberellic acid solution, the amount of GA produced by the PPFM isolates was calculated and expressed as µg ml^{-1} broth[15].

RESULTS

Isolation of PPFMs from *Capsicum anuum*

Pink pigmented facultative methylobacteria (PPFM) were isolated from surfaces of leaf samples, roots and rhizosphere soils of *C .anuum,* collected from major Chilli growing areas of North Karnataka. As many as 200 isolates were isolated, purified and given code numbers as PPFM series and maintained as the culture bank in the Department of Agricultural

Microbiology, UAS, Dharwad.

Morphological characterization

The results of the morphological characterization of PPFM isolates are presented in Table 1. All the isolates observed were Gram negative, rod shaped and motile. The colonies were pink, pale pink and dark pink in colour due to pigmentation (Table 1).

Production of growth promoting substances by PPFM

Out of 200 isolates obtained, 30 isolates were found to produce measurable quantity of Plant growth promoting substances like IAA and GA. The production of IAA and GA varied with the isolates, and the results are presented in Table 2. The highest indole acetic acid production was recorded in PPFM6 (19.77 µg/ ml of culture filtrate) followed by PPFM170 (13.21 µg/ ml). The lowest indole acetic acid production was observed in PPFM20 (0.14 µg /ml) and PPFM189 (0.17 µg/ ml). The GA production was maximum (128.28 µg/ ml of culture filtrate) in PPFM6 followed by PPFM170 (120.42 µg/ ml) while the lowest GA production was observed in PPFM7 (4.77 µg /ml).

DISCUSSION

The functional diversity of all the native PPFM isolates was analysed in terms of IAA and GA production in order to screen and select the most efficient PPFM isolates, for further intervention studies with *C. anuum* plants. In the present study, 30 isolates of PPFM were known to produce IAA and GA.

Table 1. Different habitats and morphological characterisation of PPFM isolates

S. No.	Isolates	Habitat	Cell shape	Motility	Gram reaction	Pigmentation
1	PPFM1	Phyllosphere	Rod	Positive	Negative	Pale pink
2	PPFM6	Phyllosphere	Rod	Positive	Negative	Dark pink
3	PPFM8	Root endophyte	Rod	Positive	Negative	Medium pink
4	PPFM19	Phyllosphere	rod	Positive	Negative	Medium pink
5	PPFM20	Rhizosphere soil	Rod	Positive	Negative	Pale pink
6	PPFM35	Root endophyte	Rod	Positive	Negative	Pale pink
7	PPFM38	Root endophyte	Rod	Positive	Negative	Medium pink
8	PPFM43	Phyllosphere	Rod	Positive	Negative	Dark pink
9	PPFM46	Phyllosphere	Rod	Positive	Negative	Dark pink
10	PPFM47	Root endophyte	Rod	Positive	Negative	Dark pink
11	PPFM52	Root endophyte	Rod	Positive	Negative	Pale pink
12	PPFM55	Rhizosphere soil	Rod	Positive	Negative	Medium pink
13	PPFM58	Rhizosphere soil	Rod	Positive	Negative	Pale pink
14	PPFM62	Phyllosphere	Rod	Positive	Negative	Medium pink
15	PPFM65	Root endophyte	Rod	Positive	Negative	Medium pink
16	PPFM70	Root endophyte	Rod	Positive	Negative	Pale pink
17	PPFM72	Root endophyte	Rod	Positive	Negative	Pale pink
18	PPFM77	Phyllosphere	Rod	Positive	Negative	Dark pink
19	PPFM83	Phyllosphere	rod	Positive	Negative	Medium pink
20	PPFM85	Root endophyte	Rod	Positive	Negative	Pale pink
21	PPFM86	Root endophyte	Rod	Positive	Negative	Medium pink
22	PPFM99	Phyllosphere	Rod	Positive	Negative	Dark pink
23	PPFM111	Phyllosphere	Rod	Positive	Negative	Medium pink
24	PPFM128	Root endophyte	Rod	Positive	Negative	Dark pink
25	PPFM140	Phyllosphere	Rod	Positive	Negative	Medium pink
26	PPFM154	Root endophyte	Rod	Positive	Negative	Dark pink
27	PPFM155	Root endophyte	Rod	Positive	Negative	Dark pink
28	PPFM169	Phyllosphere	Rod	Positive	Negative	Medium pink
29	PPFM170	Phyllosphere	Rod	Positive	Negative	Dark pink
30	PPFM189	Rhizosphere soil	Rod	Positive	Negative	Medium pink

Table 2. Production of plant growth hormones by PPFM isolates

S. No.	Isolates	IAA µg/ml	GA µg/ ml
1	PPFM1	2.16	35.13
2	PPFM6	19.77	128.28
3	PPFM8	4.07	44.73
4	PPFM19	2.26	40.07
5	PPFM20	0.14	19.93
6	PPFM35	11.04	108.36
7	PPFM38	7.08	88.69
8	PPFM43	0.94	12.87
9	PPFM46	4.79	46.07
10	PPFM47	6.47	80.55
11	PPFM52	1.86	39.27
12	PPFM55	0.27	20.35
13	PPFM58	0.55	12.71
14	PPFM62	2.07	18.06
15	PPFM65	7.13	89.87
16	PPFM70	0.71	4.77
17	PPFM72	1.27	16.06
18	PPFM77	2.80	48.13
19	PPFM83	0.54	13.47
20	PPFM85	1.27	30.10
21	PPFM86	6.08	39.26
22	PPFM99	9.84	104.53
23	PPFM111	4.45	52.57
24	PPFM128	0.26	10.70
25	PPFM140	9.11	100.03
26	PPFM154	0.80	36.02
27	PPFM155	6.24	47.97
28	PPFM169	0.66	28.15
29	PPFM170	13.21	120.42
30	PPFM189	0.17	7.20
	S.Em±	0.10	0.26
	CD (0.01)	0.36	0.97

The first report on the production of indole acetic acid in significant amount by four different methylotrophs was observed[7]. Variability among PPFM isolates in IAA production (0.14 to 25.12 µg/ml) was observed[16]. The production of IAA by PPFM ranging was from 9.04 µg/ml to 28.15 µg/ml [17]. Gibberellic acid is another group of plant growth regulators, which act by modifying the plant morphology[18]. It is known to induce the uptake of minerals like K and Ca, increase the chlorophyll content, soluble sugars and protein content of

a) Serial dilution technique

b) Leaf imprinting technique

Fig. 1.

plants. The production of gibberellic acid by eight of the PPFM isolates tested and the GA production varied from 10.9 µg /ml to 106.97 µg/ ml [11]. PPFM isolates produced GA ranging from 1.33 to 6.83 µg/ 25ml [16].

CONCLUSION

In the present study, as many as 200 PPFM isolates were isolated from surfaces of leaf, roots and rhizosphere soil samples of *C .anuum* on AMS medium, which is a selective medium for PPFMs, among these 30 isolates were known to produce significant amount of plant growth promoting substances like IAA and GA. These isolates were preserved for further use to study their influence on the growth and yield of chilli under field conditions.

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