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Research Article

PRODUCTION OF INDOLE ACETIC ACID, CYTOKININS AND GIBBERELIC ACID BY PINK PIGMENTED FACULTATIVE METHYLOTROPHS

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ABSTRACT

In the present study, PPFMs were isolated from phyllosphere of fourteen plants by leaf imprinting and serial dilution techniques. The fourteen isolates were characterized by morphological, biochemical and molecular methods. The PPFM isolates were screened based on the effect on germination and seedling characters of cowpea cv. CO(CP)7. PPFM inoculation enhanced the germination, seedling length, vigour index, biomass, chlorophyll and soluble protein content of cowpea. Based on this study, nine best isolates including the reference strain *M. extorquens* AM1 were selected for further studies. The selected PPFM isolates were screened based on plant growth hormone production. The isolates produced *trans*-zeatin in amounts ranging from 22.04 to 117.32 ng l^{-1} of culture filtrate. IAA production ranged from 0.14 to 4.69 µg ml^{-1} of culture filtrate in the absence of the precursor tryptophan and 0.97 to 8.32 µg ml^{-1} in the presence of tryptophan. All isolates could produce gibberellic acid in amounts ranging from 39.33 to 123.0 µg ml^{-1} of culture filtrate. The primers specific for *ipt* gene encoding isopentenyl transferase, the key enzyme in direct synthesis of cytokinins gave amplification in the isolate PPFM-Ph. The *trans*-zeatin riboside content of four best isolates (PPFM-As, PPFM-Ph, PPFM-Pt and *M. extorquens* AM1) as estimated by ELISA ranged from 11.22 to 37.07 ng l^{-1} of culture filtrate.

KEYWORDS: PPFM, IAA, *trans*-zeatin, gibberellic acid.

INTRODUCTION

Pink-pigmented facultative methylotrophs (PPFMS) are ubiquitous inhabitants of phyllosphere and rhizosphere of plants. PPFM-plant symbiosis is one of the less conspicuous symbioses, but the ability of these bacteria to supply plant growth hormones to the host has attracted much attention in recent years. The interaction between microorganisms and higher plants considerably affects the physiological activities of the latter (Kalyaeva *et al.,* 2001). It is speculated that plants have established symbiotic relations with aerobic methylotrophic bacteria which are ubiquitous and abundant in the plant rhizosphere and phyllosphere (Trotsenko *et al.,* 2001). Aerobic methylotrophic bacteria utilize methane or its oxidized or substituted derivatives as sources of carbon and energy (Anthony, 1986). Facultative methylotrophy of PPFMs is partly responsible for the maintenance of their relationships with plants (Corpe and Rheem, 1989). The ability of PPFMs to promote plant growth under *in vitro* and *in vivo* conditions is well documented (Kalyaeva *et* al., 2001 and 2003; Madhaiyan *et al*., 2004 and 2005). The plant growth promoting effect of PPFMs is attributed to the production of phytohormones and vitamins by these bacteria. PPFMs produce cytokinins (Ivanova *et al*., 2000) and auxins (Ivanova *et al*., 2001; Doronina *et al*., 2002 and Omer *et al*., 2004b) in the culture medium. In the present study, the potential of PPFMs to produce hormones like cytokinin, indole acetic acid and gibberellins was evaluated.

MATERIALS AND METHODS

1. Isolation of facultative methylotrophs from phyllosphere regions

Pink pigmented facultative methylotrophs were isolated from fourteen plants by leaf imprinting technique (Corpe, 1985). Methanol mineral salts (MMS) or ammonium mineral salts (AMS) medium (Whittenbury *et al*., 1970) was sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. Filter sterilized vitamin solution (Colby and Zatman, 1973) was added, along with 0.5 percent v/v sterile methanol. The pH of the medium was adjusted to 7.0. Solid medium (MMS agar) was prepared by the addition of 1.5 – 2.0 per cent agar before autoclaving. The upper and lower surface imprints of fresh leaf samples were made separately on the solidified media and incubated at 28 ± 2° C for 5 days. Characteristic pink colored colonies were isolated and purified by streak plate method. Since the growth was reported to be more luxuriant with a deeper pigmentation on glycerol-peptone (GP) medium (Green, 1992), GP agar was used for sub culturing and storing for a longer time under refrigerated conditions.

2. Estimation of plant growth hormones from culture filtrate of PPFMs

2.1. Cytokinin production

2.1.1. Extraction of cytokinin

The bacterial isolates were grown in ammonium mineral salts medium for ten days at room temperature in a rotary shaker. After incubation, the culture broth was centrifuged at 10000 rpm for 15 min to pellet the cells. The cell-free culture suspension was acidified to pH 2.9 with 0.1N HCl and extracted thrice with equal volumes of diethyl ether to remove auxins. The pH of the aqueous phase was adjusted to 7.0 with 1N ammonium hydroxide and extracted thrice with equal volumes of n-butanol. The organic fractions were pooled and evaporated. The residue was dissolved in 3 ml of HPLC grade methanol. The methanol fraction was filtered through 0.2 µm bacterial filter and used for high performance liquid chromatograph analysis.

2.1. 2. Estimation of *trans*-zeatin production by HPLC (Tien *et al*., 1979)

A volume of 5µl of sample was injected into the HPLC. Analysis was performed using a 5 µm particle size reverse phase (C_{18}) column (4.6 x 250 mm) with a solvent gradient of 30 per cent methanol in water at a flow rate of 1.0 ml min^{-1}. Quantification of cytokinin compounds in the sample was done by comparison of retention time with peak height of *trans*-zeatin (Sigma grade). The cytokinin compounds were quantified by integrating the areas under the peaks using UV detector at 254 nm.

2.2. IAA production (Gordon and Weber, 1951 and Ivanova *et al.*, 2001)

2.2.1. Extraction of IAA

AMS broth added with L- tryptophan (100 mg l^{-1}) was prepared and 100 ml quantities were dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized at 15 psi for 20 min. One ml of the inoculum (10^9 cfu $m1^{-1}$) of PPFM isolate was added and incubated at room temperature in a shaker for 10 days. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After incubation, the culture broth was centrifuged at 10000 rpm for 15 min and the supernatant was taken. The pH of the supernatant was adjusted to 2.8. Extraction was done thrice using equal volumes of diethyl ether at 4°C. Organic fractions were pooled and evaporated in dark. The residue was dissolved in 2 ml methanol

2.2.2. Quantitative estimation of IAA

A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by 4 ml of Salper's reagent and incubated in darkness for 1 h at 28°C. The intensity of the pink colour developed was read in spectrophotometer at 535 nm. The quantity of IAA in the sample was determined by referring to a standard graph prepared with chemical grade indole-3-acetic acid.

2.3. Gibberellic acid (GA) production

2.3.1. Extraction of gibberellins (Tien *et al.*, 1979)

AMS broth was prepared and 50 ml quantities were dispensed in 250 ml Erlenmeyer flasks. The flasks were sterilized at 15 psi for 20 min. One ml of the inoculum of PPFM isolates with a population density of 10^9 cfu $m1^{-1}$ was added to each flask and incubated for 10 days at room temperature in a shaker. The culture broth was centrifuged for 15 min at 10,000 rpm and 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 0.1N HCl 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 15 ml of methanol.

2.3.2. Spectrophotometric estimation of gibberellins (Holbrook *et al.*, 1961)

Two ml of zinc acetate solution was added to fifteen ml of methanol fraction. 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% HCl and the mixture was incubated at 20°C for 75 min. The blank was treated with 5 % HCl. The absorbance was measured at 254 nm in a spectrophotometer.

RESULTS AND DISCUSSION

1. Isolation of facultative methylotrophs from phyllosphere regions

Leaves of fourteen plants were collected from in and around Tamil Nadu Agricultural University campus, Coimbatore. The native pink-pigmented methylotrophic bacteria (PPFM) were isolated from the leaves by leaf imprinting technique on ammonium mineral salts medium supplemented with 0.5 percent methanol. Pink coloured colonies appeared on the agar plates after 5 to 7 days of incubation. These colonies were purified by streak plate method and maintained in glycerol-peptone agar slants for further studies.

PPFMs are abundant and ubiquitous and have been isolated from almost all plants tested to date. Their populations range from 10^4 to 10^7 cfu g^{-1} fresh weight of plant tissue, with the highest numbers present on actively growing meristematic tissue (Holland and Polacco, 1992; Hirano and Upper, 1992 and Holland, 1997).

2. Estimation of plant growth hormones in culture filtrates of PPFMs

2.1. *trans*-zeatin production

The nine best isolates selected from the germination and vigour index test conducted using cowpea seeds were tested for *trans*-zeatin production by HPLC analysis of n-butanol extract of culture supernatant. All isolates were found to produce the cytokinin *trans*-zeatin in varying amounts. The *trans*-zeatin production ranged from 22.04 ng l^{-1} of culture filtrate in the isolate PPFM-Te to 117.32 ng l^{-1} of culture filtrate in the isolate PPFM-Ph. The *trans*-zeatin production by the isolates PPFM-As (97.76 ng l^{-1} of culture filtrate) and PPFM-Pt (93.28 ng l^{-1} of culture filtrate) were statistically on par. The reference strain *M. extorquens* AM1 produced 58.88 ng of *trans*-zeatin l^{-1} of culture filtrate (Table 1). The best isolates were PPFM-Ph, PPFM-As, PPFM-Pt and *M. extorquens* AM1

Cytokinins have been previously identified from culture filtrates of PPFMs. PPFM isolates from soybean, maize, barley and *Arabidopsis* were found to contain zeatin and zeatin riboside in amounts ranging from 50 to 400 ng g^{-1} dry biomass (Long *et al.,* 1997). Ivanova *et al.* (2000) identified cytokinin activity in *Methylobacterium mesophilicum* VKMB-2143 and *Methylovorus mays* VKMB-2221. Koenig *et al.* (2002) reported the presence of *trans*-zeatin (22-111 ng l^{-1} of culture filtrate) in PPFM isolates of some plants. *Trans*-zeatin riboside was also detected in the culture filtrates of the isolates in smaller quantities. Reddy *et al.* (2002) reported that PPFM isolates of tropical plants produce 33 to 238 ng of *trans*-zeatin per litre of culture filtrate.

2.2. IAA production

The isolates under study were able to synthesize IAA both in the presence and absence of the precursor tryptophan. In the absence of tryptophan, the isolate PPFM-Pt produced maximum IAA (4.69 µg ml^{-1} of culture filtrate) followed by the isolate PPFM-As (3.55 µg ml^{-1} of culture filtrate). The isolate PPFM-Ms produced the least amount of IAA (0.14 µg ml^{-1} of culture filtrate) in the absence of tryptophan. The reference strain *M. extorquens* AM1 produced 1.55 µg of IAA ml^{-1} in the absence of tryptophan.

In general, it was found that addition of tryptophan enhanced the IAA production. In the presence of the precursor, tryptophan also, a similar trend was observed. IAA production was maximum in the isolate PPFM-Pt (8.32 µg ml^{-1} of culture filtrate) when tryptophan was provided in the medium. It was followed by the isolate PPFM-As (6.87 µg ml^{-1} of culture filtrate). The reference strain *M. extorquens* AM1 synthesized 3.14 µg of IAA ml^{-1} of culture filtrate in the presence of tryptophan. The isolate PPFM-Ms produced the least amount of IAA (0.97 µg of IAA ml^{-1} of culture) in the presence of tryptophan also and was on par with the isolates PPFM-Pg, PPFM-Te and PPFM-Jg (Table 1).

The present study is supported by earlier findings that PPFMs can produce auxins (Ivanova *et al.,* 2001; Doronina *et al.,* 2002 and Omer *et al.,* 2004b). Obligate and facultative methylotrophic bacteria were found to produce auxins particularly indole-3-acetic acid (IAA) in amounts ranging from 3-100 µg ml^{-1} of culture liquid. The isolates could also produce indole-3-lactic acid in trace amounts. The addition of tryptophan to the culture medium enhanced the synthesis of indole compounds (Ivanova *et al.,* 2001). PPFM isolates from vegetable crops were shown to produce 3.48 to 8.77 µg of IAA ml^{-1} of culture filtrate in the presence of tryptophan and 1.31 to 4.88 µg of IAA ml^{-1} of culture filtrate in the absence of tryptophan. The variation in the ability of methylobacteria to synthesize auxins and the positive effect of the precursor tryptophan on auxin production has been reported (Omer *et al.,* 2004b).

2.3. Gibberellic acid production

The isolates showed variability in the quantity of gibberellic acid (GA) produced. All isolates could produce GA in amounts ranging from 39.33 to 123.0 µg ml^{-1} of culture filtrate. The highest concentration (123.0 µg ml^{-1}) of GA was found in the isolate PPFM-As. It was followed by the isolate PPFM-Pt (91.33 µg ml^{-1} of culture filtrate). The reference strain *M. extorquens* AM1 produced 53.33 µg of GA ml^{-1} of culture filtrate. The GA production by the isolate PPFM-Ph was 64.33 µg ml^{-1} of culture filtrate (Table 1).

Koenig *et* al. (2002) found that the *miaA* mutant of *M. extorquens*, in whose medium there was no detectable cytokinin, stimulated germination at a level indistinguishable from the wild type. They postulated that PPFMs may produce or stimulate gibberellins which play an important role in the release of seeds from dormancy. Gibberellic acid production has been reported in PPFMs by Sheela *et al*. (2013).

Table 1: Estimation of plant growth hormones in culture filtrate of PPFMs.

Sl. N.	Isolate	*trans*-zeatin content	IAA content (µg ml^{-1})		Gibberellic acid content
		($ng\ l^{-1}$)	Without tryptophan	With tryptophan	(µg ml^{-1})
1	PPFM-As	97.76	3.55	6.87	123
2	PPFM-Cm	41.32	0.41	1.21	39.33
3	PPFM-Jg	70.36	0.17	1.32	69.67
4	PPFM-Te	22.04	0.68	1.86	59.57
5	PPFM-Pt	93.28	4.69	8.32	91.33
6	PPFM-Ms	48.92	0.14	0.97	64.67
7	PPFM-Pg	42.64	0.16	1.08	69.67
8	PPFM-Ph	117.32	3.07	6.96	64.33
9	*M. extorquens* AM_1	58.88	1.55	3.14	53.33
SEd		5.894	0.168	0.335	6.441
CD (0.5%)		12.383	0.353	0.705	13.533

CONCLUSION

The PPFM isolates were found to synthesize plant growth hormones. The isolates produced *trans*-zeatin in amounts ranging from 22.04 to 117.32 ngl^{-1} of culture filtrate. IAA production in the selected isolates ranged from 0.14 to 4.69 µg ml^{-1} of culture filtrate in the absence of tryptophan and 0.97 to 8.32 µg ml^{-1} in the presence of tryptophan. All isolates could produce gibberellic acid in amounts ranging from 39.33 to 123.0 µg ml^{-1} of culture filtrate.

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