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Plant Growth Promoting Activity of Pink Pigmented Facultative Methylotroph - *Methylobacterium extorquens* MM2 on *Lycopersicon esculentum* L.

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ABSTRACT

Pink pigmented facultative methylotrophs (PPFMs) are diversified group of microorganisms that promote plant growth by producing indole acetic acid (IAA) and cytokinins. The objective of the present study is to isolate and characterize methylotrophic bacteria from the phyllosphere of mustard plant (*Brassica niagra*) and to study their plant growth promotion in tomato (*Lycopersicon esculentum* L.). Bacteria were isolated from the phyllosphere and the conserved *mxaF* gene sequence was amplified. Production of IAA was determined by High Performance Thin Layer Chromatography (HPTLC) and further quantified by spectrophotometric analysis. Effect of seed bacterization with the bacterial isolate MM2 was studied for percentage seed germination, root and shoot length. Based on NCBI BLAST search of the *mxaF* sequence obtained, the bacterial isolate was identified as *Methylobacterium extorquens* MM2. The amount of IAA produced by *M. extorquens* MM2 was 6.16 µg/ml. *M. extorquens* MM2 significantly increased seed vigour index (SVI) in tomato plants. The SVI of the treated plant was 1462.56 ± 83.96 as compared to the control, 1019.94 ± 113.61. Foliar application of *M. extorquens* MM2 also contributed a substantial impact on growth of tomato plant. The bacterial isolate *M. extorquens* MM2 thus can be employed in the crops for sustainable agriculture.

1. INTRODUCTION

Phyllosphere harbours a diverse group of microorganisms and the interaction between the plant and microbes greatly affect the physiological activities of the plant. Pink pigmented facultative methylotrophs (PPFMs) are the prime inhabitants of phyllosphere region of wide variety of plant species [1, 2]. PPFMs are classified as α-proteobacteria, capable of growing even on single carbon compounds such as methanol and methylamine [3]. *Methylobacterium* are able to oxidize methanol in presence of methanol dehydrogenase (MDH) encoded by *mxaF* gene [4]. The presence of highly conserved *mxaF* gene in *Methylobacterium* is a molecular marker for identification, characterization and to study extent of diversity [5]. In addition to that, the widespread occurence of carotenoids in PPFMs also serve as an important taxonomic marker for the identification of the isolates. Many strains of *Methylobacterium* sp. promote growth of the host plant by producing indole acetic acid (IAA). They synthesize a variety of auxins and cytokinins that are utilized by the host plants for growth and development. Many researchers reported the role of PPFMs in enhancing plant growth, seed germination, seed vigour index, plant yield and systemic resistance of the plant [6]. Methylotrophic bacteria on plant surface act in a symbiotic relationship with the host plants as they get benefited by methanol emitted from plants as a byproduct of pectin degradation [7].

Based on the plant-microbe interaction *Methylobacterium* sp. are classified as biostimulator, biofertilizer and biocontroller as they directly influence the plant growth by producing phytohormones especially auxins, supply nutrients to the plants and induce systemic resistance in the plants against phytopathogens [8]. In recent years, *Methylobacterium* received considerable attention for industrial and agricultural applications [9]. Methylotrophs biotransform chemical substrates into valuable products with enhanced economic value.

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Their ability to produce IAA, to enhance seed germination, plant development, vitamin production, ACC deaminase activity and siderophores production makes them promising tool for sustainable agriculture [10]. The present study is focused on the isolation of PPFMs from the phyllosphere of *Brassica niagra* and to establish their plant growth promotion in tomato plant (*Lycopersicon esculentum* L.) by enhancing the production of IAA.

2. MATERIALS AND METHODS

2.1 Isolation and morphological identification of PPFMs

Leaves from mustard plant (*Brassica niagra*) were collected from Nilgiri district, Tamilnadu, India. Ammonium mineral salt (AMS) agar medium supplemented with 0.5 % methanol (C_1 carbon substrate) and 0.1 % cycloheximide (antifungal agent) was used for selective isolation of PPFMs. The adaxial surface of the leaf was imprinted on the solidified agar medium using leaf imprint method and incubated at 27 °C for 7 days. The bacteria were isolated from pink coloured colonies and selected based on the intensity of the colour. Morphology of the bacterial isolate was observed using Scanning Electron Microscope (SEM) [11].

2.2 Molecular Characterization of bacterial isolate

The genomic DNA of the bacterial isolate was extracted by using phenol followed by ethanol precipitation [12]. The presence of methanol dehydrogenase gene (*mxaF*) was detected by PCR amplification using the *mxaF* specific primers F1003 (5′GCGGCACCAACTGGGGCTGGT3′) and R1561 (5′GGGAGCCCTCCATGCTGCCC3′). The PCR product was analyzed by agarose gel electrophoresis (1.5% agarose) and further sequenced at the Macrogen Inc (South Korea). The obtained sequence was subjected for NCBI GenBank database search. Phylogenetic tree was constructed by maximum parsimony method using MEGA 5.0 software [13, 14].

2.3 Determination of IAA Production

Qualitative and quantitative analysis of IAA production by the bacterial isolate was investigated. IAA was extracted from the bacterial culture according to the method described by Goswami *et al*. 2015. Production of IAA was determined by High performance thin layer chromatography (HPTLC). The amount of IAA produced by the bacterial isolate was determined spetrophotometrically (530 nm) using Salkowski's reagent. The amount of IAA produced by the bacterial isolate was quantified using IAA as standard [15, 16].

2.4 Plant growth promotion in tomato

2.4.1 Determination of Seed vigour index (SVI)

Seed vigour index (SVI) indicates the ability to germinate seeds and increase in root and shoot length of seedlings. Seed bacterization improves the SVI [17]. Seven days old bacterial culture was used for the determination of SVI. Sodium hypochlorite (0.5 %) was used to sterilize the surface of the tomato seeds. The seeds were treated with bacterial suspension (10^8 CFU/ml) and incubated overnight [18]. After incubation, 15 seeds were placed in equidistance manner on 0.7 % solidified agar plates and further incubated for 6 days. A control experiment was performed without seed bacterization. All the experiments were carried out for 3 times (n=3). Percentage germination, root and shoot length of the seedlings were calculated. SVI was determined using the formula:

SVI= (Root length + shoot length) X % germination.

2.4.2 Pot experiment

Plant growth promotion of bacterial isolate could be exploited for the agriculture application. In the present study, the effect of bacterial isolate on the growth of tomato (*L. esculentum* L.) plant was evaluated. The germinated seedlings treated with bacterial suspension (10^8 CFU/ml) were sown in the pots containing sterile soil ($3/4^{th}$ of the pots were filled with soil). The seeds treated with uninoculated broth served as control. The bacterial suspension (10^8 CFU/ml) was sprayed on the phyllosphere of the plant after 21 days of sowing and observed for its growth promoting traits. Root length and shoot length were measured after 33 days of growth [18]. All the experiments were conducted in triplicate.

3. RESULTS

3.1 Isolation of PPFMs

Tiny, pink colored colonies were observed on AMS medium after 7 days of incubation. Based on the colony morphology and intensity of colour of the colonies, bacterial isolate MM2 was selected for further study. Rod shaped bacterial cells were observed in scanning electron micrographs (Figure 1).

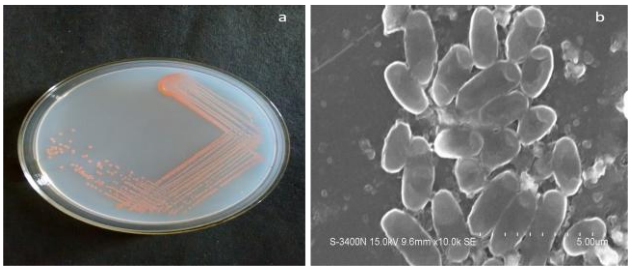

Fig. 1: Isolation and characterization of Methylotrophic bacteria from the phyllosphere of mustard plants (a) PPFM strains isolated from mustard leaves, (b) SEM micrograph of *Methylobacterium extorquens* MM2

3.2 Molecular characterization of Methylotrophs

PCR amplification of *mxaF* gene and agarose gel electrophoresis confirmed the presence of methanol dehydrogenase gene with a size of 550-560 bp (Figure 2). NCBI BLAST search analysis of *mxaF* gene sequence showed 99 % similarity to *Methylobacterium extorquens*. The bacterial isolate was named as *Methylobacterium extorquens* MM2. Phylogenetic analysis of *mxaF* gene sequence confirmed its relationship with other Methylobacterium species (Figure 3).

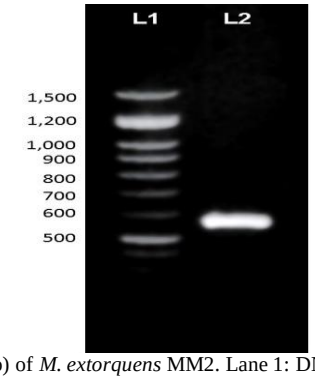

Fig. 2: *mxaF* gene amplification (550 bp) of *M. extorquens* MM2. Lane 1: DNA Ladder; Lane 2: *M. exteorquens* MM2

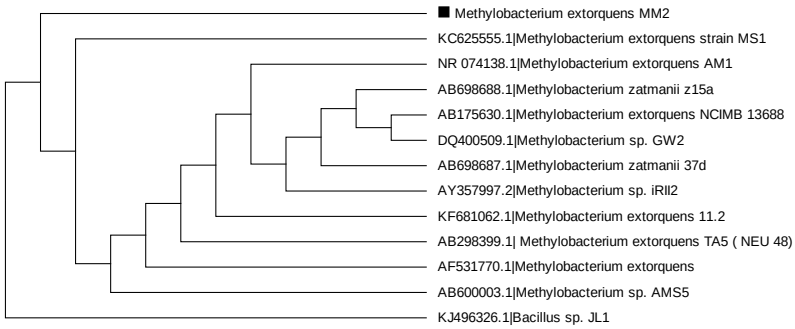

Fig. 3: Neighbour joining Phylogenetic tree constructed based upon *mxaF* gene sequences showing the position of *M. extorquens* MM2 using maximum parsimony method (MEGA 4.0).

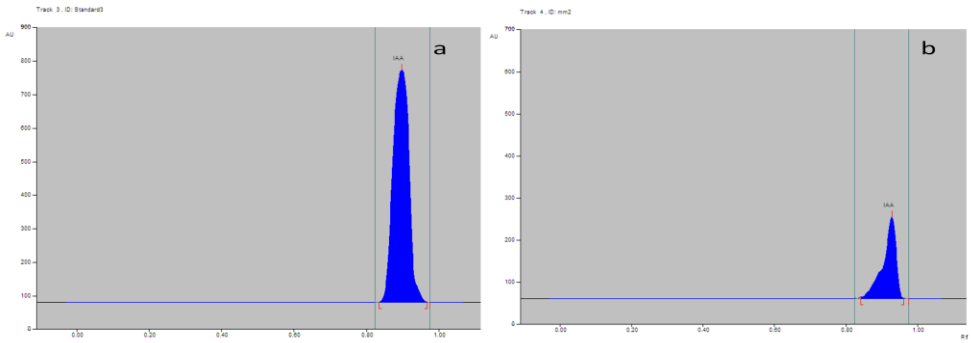

Fig. 4: Qualitative analysis of production of IAA of *M. extorquens* MM2 by HPTLC technique (a) Sharp peak of standard IAA (1000 µg/ml) (b) MM2 peak corresponding the standard compound's R_f.

3.3 Determination of IAA production

Production of IAA by *M. extorquens* MM2 was confirmed by HPTLC analysis. A sharp peak with an R_f of 0.91 was observed which is similar to that of standard IAA (Figure 4). The amount of IAA produced by cell-free culture filtrate of *M. extorquens* MM2 was 6.16 µg/ml.

3.4 Plant growth promotion by *M. extorquens* MM2

3.4.1 Determination of seed vigour index (SVI)

Bacterization of tomato seeds with *M. extorquens* MM2 significantly improved the growth promoting traits such as percentage germination, seedling length with a concomitant increase in SVI compared to the control. Seed bacterization with *M. extorquens* MM2 increased the seed germination to 97.7 ± 1.49 % as compared to 95.5 ± 1.20 % in control. Significant increase in seedling vigour from 1019.94 ± 113.61 to 1462.56 ± 83.96 was observed in the seeds treated with *M. extorquens* MM2 compared to control (Table 1).

Table 1 Effect of seed bacterization with *M. extorquens* MM2 on plant growth promoting traits (germination percentage, root and shoot length) as compared to the control.

Plant growth promotion	Control	Treated with *M. extorquens* MM2
Seed Germination (%)	95.5 ± 1.20	97.7 ± 1.49
Root Length (cm)	5.22 ± 0.48	6.87 ± 0.97
Shoot Length (cm)	5.46 ± 0.41	8.09 ± 0.24
Seedling Length (cm)	10.68 ± 0.9	14.97 ± 1.20
Seed vigour index	1019.94 ± 113.61	1462.56 ± 83.96

3.4.2 Pot Experiment

Seed bacterization and foliar application of *M. extorquens* MM2 significantly enhanced root and shoot length and number of leaves per plant. Treatment with *M. extorquens* MM2

resulted in the enhancement in root length to 9.66 ± 0.68 cm in tomato plants compared to the root length of control, 7.23 ± 0.49 cm. In terms of shoot length, similar significant increase was recorded in *M. extorquens* MM2 inoculated (42.43 ± 4.17 cm) plants over the control (34.96 ± 4.22 cm) (Table 2, Figure 5).

Table 2 Effect on plant growth promoting traits of *L. esculentum* plants on treatment with *M. extorquens* MM2.

Treatment	Root Length (cm)	Shoot length (cm)	No. of Leaves
Control	7.23 ± 0.49	34.96 ± 4.22	38.33 ± 3.21
***M. extorquens* MM2**	9.66 ± 0.68	42.43 ± 4.17	53.66 ± 3.05

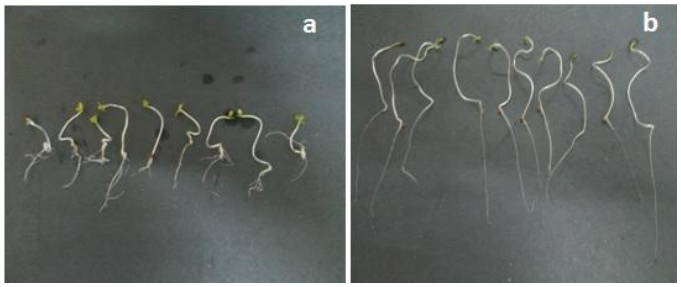

Fig. 5: Effect of *M. extorquens* MM2 on seed germination and seedling length of tomato (*L. esculentum* L.). (a) seedling length of untreated seeds (Control); (b) seedling length of *M. extorquens* MM2 treated seeds

4. DISCUSSION

PPFMs are highly diversified group of microorganisms abundantly present on the phyllosphere region of several plant species and enhance the plant growth by producing a wide variety of phytohormones [5, 6]. *Methylobacterium* are symbiotically associated with host plant and regulates host plant growth promotion by producing a number of phytohormones like IAA, cytokinins [3, 19]. In the present study, PPFM was isolated from mustard leaves. The *mxaF* gene of the bacterial isolate MM2 was amplified, sequenced and identified as *Methylobacterium extorquens*. The bacterium was investigated for the production of indole acetic acid and its relation with plant growth promotion was established. Microbes utilize methanol dehydrogenase (MDH) enzyme for the degradation of methanol encoded by *mxaF* gene which can be employed as marker gene for the identification of *Methylobacterium* [8].

In the present study production of IAA by bacterial isolate was identified by HPTLC. The amount of IAA produced by the *M. extorquens* MM2 was determined as 6.16 µg/ml. Previous studies reported that the amount of IAA produced by *Methylobacterium* sp. Mb10, *Methylobacterium* sp. Mb14 and *Methylobacterium* sp. HSC5 was 1.13, 2.28 and 2.40 µg/ml respectively [20]. The results indicate the significantly higher production of IAA by *M. extorquens* MM2. Effect of seed bacterization enhanced the seedling vigour index (1462.56 ± 83.96) over the control (1019.94 ± 113.61) which was significantly higher than the report by Meena *et al*. 2012 with a SVI value of 1022.3 for the *Methylobacterium* sp. (NC4) isolated from sugarcane (*Saccharum officinarum*) [5]. Foliar application of *M. extorquens* MM2 significantly improved root and shoot length and number of leaves in tomato plants. *M. extorquens* MM2 can be further studied for its application in plant growth promotion for sustainable agriculture in economically important crops.

5. CONCLUSION

Different colonies of pink pigmented facultative methylotroph (PPFM) were isolated from the phyllosphere of mustard leaf. Among the bacterial isolates, MM2 was further studied and identified as *M. extorquens*. *M. extorquens* MM2 significantly increased the production of IAA, which attributed towards substantial increase in plant growth promotion. *M. extorquens* MM2 can thus be further employed in sustainable agriculture.

6. CONFLICT OF INTEREST

The authors declare that research article does not contain any conflict of interest.

7. ACKNOWLEDGEMENT

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