

Isolation and Characterization of Tomato Leaf Phyllosphere *Methylobacterium* and their Effect on Plant Growth

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ABSTRACT

Phyllosphere methylophilic bacteria from tomato leaves were isolated using selective Ammonia Mineral Salt (AMS) medium. Totally thirty two epiphytic bacterial isolates were selected based on their colony morphology and distinct pigmentation. All the isolates tested were Gram negative, rod shaped and positive for urease, catalase and oxidase activities. The preliminary screening of five randomly selected strains under *in vitro* for plant growth promoting characteristics and evaluated for their beneficial effects on the early growth of tomato showed positive for nitrogen fixation, ACC deaminase, siderophore and phytohormone productions were chosen for further studies. An *in vitro* experiment was conducted with 4 selected *Methylobacterium* spp. to evaluate their effect on tomato seed germination and vigor index. Among the isolates tested, Mt-Tm-13 had significantly increased the seed germination percentage (100%) earliness in radical emergence and vigor index. Plant inoculation studies indicated that *Methylobacterium* spp. strains provided a significant beneficial effect to the plants. In pot culture experiment, foliar application of *Methylobacterium* spp. (1% v/v concentration) showed that the inoculated plants had significant plant growth, biomass as well as yield attributes. The present study suggests that the *Methylobacterium* isolates may be used as bio-inoculants for sustainable tomato cultivation.

Keywords

Methylobacterium sp.,
Tomato, Phyllosphere
bacteria, Plant growth
promotion.

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Introduction

The criticality of the interaction between plants and microorganisms to agricultural output is increasingly recognized. The role of microorganisms in plant growth promotion, nutrient management and disease control is well known. These beneficial microorganisms colonize the rhizosphere/endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Saxena *et al.*, 2005). Aerial part of plants are termed as phyllosphere, and the

inhabitants are called epiphytes. The overall microbiota in this ecosystem is thus sufficiently large to influence their hosts at the level of the individual plants. Bacteria are by far the most numerous colonists of leaves, often being found in numbers averaging 10^6 to 10^7 cells cm^{-2} (up to 10^8 cells g^{-1}) of leaf (Andrews and Harris, 2000). Clearly, in aggregate, these bacteria are sufficiently numerous to contribute in many processes of importance to global processes, as well as to

the behavior of the individual plants on which they live.

It is believed that the phyllosphere is dominated by a few bacterial genera. *Pseudomonas*, *Xanthomonas*, *Bacillus*, *Erwinia*, and *Methylobacterium* spp. are detected most frequently (Thompson *et al.*, 1993). However, many more taxa are present at low abundance and cultivation-independent studies indicated that bacterial communities on leaf surfaces can be expected to be much more complex than previously thought (Lambais *et al.*, 2006). The vast majority of phyllosphere bacteria is believed to live as commensal organisms without harmful or beneficial effects for the host plant. Little is known about the role of these abundantly occurring bacteria in the phyllosphere ecosystem. On the contrary, a number of bacterial isolates are known to exhibit a plant-beneficial effect. An example for beneficial phyllosphere bacteria, which are known to directly stimulate plant growth, are the pink-pigmented facultative methylotrophs (PPFM).

Methylobacterium spp. colonize in the plant phyllosphere and endosphere, comprising one of the dominant groups in each of these habitats (Pirttila *et al.*, 2005). They are capable of using the plant-derived, reduced one-carbon compound methanol as sole source of carbon and energy (Abanda-Nkpwatt *et al.*, 2006, Sy *et al.*, 2005). In addition, the pink pigmentation is thought to protect the bacteria from damage by solar radiation during epiphytic growth. The association between *Methylobacterium* spp. and host plants varies from strong or symbiotic (Jourand *et al.*, 2004) to weak or epiphytic (Omer *et al.*, 2004) and to intermediate or endophytic (Lacava *et al.*, 2004). Interestingly, *Methylobacterium*-plant association in the phyllosphere is not highly genotype specific (Knief *et al.*, 2010). These bacteria evidently have an intimate

association with plants, as they promote plant growth and development by production of phytohormones auxins and cytokinins, which benefit the germination of seeds, and plant growth and development (Ivanova *et al.*, 2001) and ACC deaminase activity (Madhaiyan *et al.*, 2007). Therefore *Methylobacterium* strains may be useful for the wide range of crops as microbial agents in sustainable agriculture. Abanda-Nkpwatt *et al.*, (2006) reported about a *Methylobacterium extorquens* strain that promotes growth of various plant seedlings, while Hornschuh *et al.*, (2002) isolated two *Methylobacterium* strains that positively affect growth of *Funaria* mosses. Moreover, it was shown for several PPFMs that they produce plant hormones – auxins and cytokines – *in vitro* (Ivanova *et al.*, 2001, Omer *et al.*, 2004). This strongly suggests that plant hormones produced by phyllosphere bacteria are responsible for plant-growth promoting effects.

Tomato is one of the popular vegetables of great commercial value and is used in various forms. Among them major production countries are China, EU, India, US and Turkey, with China accounting for 31% of the total world production followed by India. In India it occupying an area of 791 thousand ha, with production of 17398 thousand tons with an average yield of 22 t/ha (Horticulture statistics data, 2014-15 Government of India). This crop is gaining importance both in developing and developed countries and efforts are being made for the quality and quantity production of this commodity (Mahajan and Singh, 2006). It is well known fact that increased dependence on agro chemicals including fertilizers has led to several ill effects on the environment and also results in decrease of soil fertility. Application of organic manure plays an important role on yield and its attributes as well as nutrient uptake and directly increase the soil physical

condition. Use of biofertilizer and organic manure in agriculture is becoming popular nowadays for not only in order to reduce the cost of chemical fertilizers but also to decrease the adverse effects of chemical fertilizers on soil and plant environment and to ensure more crop productivity.

The present study was designed to explore the various responses of tomato plants to foliar application of different *Methylobacterium* strains on tomato crops under *in vitro* conditions.

Materials and Methods

Healthy and asymptomatic 2-3 fresh leaves were collected from 60 days old tomato plants (variety PKM 1) grown in orchard of Tamil Nadu Agricultural University, Coimbatore, South India. Leaves were collected at 9:00A.M, stored in sterile propylene bags at 4°C and analyzed within 24 h after their collection. Samples were taken from 3rd leaf position from each plant were collected and cleaned under running tap water to remove debris and then air dried. Each leaf was divided into two equal parts with one part from the apex and one from the base was selected for analysis.

Tomato leaves were leaf imprinted in the Ammonia Mineral Salt (AMS) media for 1 minute and the plates were incubated at 30⁰C for 7days till a distinct pink color colony was appeared along the leaf imprinting area. From each plate, based on distinct colony morphological features bacterial colonies were chosen at random.

The isolates were maintained on AMS agar slants at 4⁰C and also as 30% glycerol stocks at -80⁰C. Biochemical characterization of the *Methylobacterium* isolates was carried out according to Bergeys Manual of Determinative Bacteriology.

Plant growth promoting traits of the isolates

Nitrogen fixing ability

Methylobacterium spp. were tested for their growth in nitrogen free medium. The bacterial isolates were grown in AMS broth without addition of NH₄Cl and incubated at 28°C for 10 days and bacterial growth was observed as qualitative evidence of the atmospheric nitrogen fixation.

The growth measurement was taken in a UV-vis spectrophotometer (Cary 50 Bio, varian) at 660 nm and expressed as – no growth (< 0.1 OD); + (0.1 to 0.3) and ++ (0.3 to 0.5).

ACC deaminase activity

Screening for ACC deaminase activity of epiphytic *Methylobacterium* sp. isolates were done as mentioned by (Penrose and Glick, 2003). All the isolates were grown in 10 ml of AMS medium incubated at 28°C at 120 rpm for 4 to 5 days and cells were harvested by centrifugation at 3000 g for 5 min, washed twice with sterile 0.1 M Tris-HCl (pH 7.5), resuspended in 1 ml of 0.1 M Tris HCl (pH 7.5) and spot inoculated on petri plates containing modified DF minimal medium supplemented with 3mM ACC as sole nitrogen source.

Plates containing only DF salts minimal medium without ACC as negative control and with (NH₄)₂SO₄ (0.2% w/v) as positive control. The plates were incubated at 28°C for 72 h. Growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as sole nitrogen source. The presence or absence of growth indicate positive for ACC deaminase activity and negative for ACC deaminase activity respectively.

Siderophore production

The production of siderophore by the bacterial isolates were performed qualitatively by plate assay (Schwyn and Neilands, 1987). Seventy two hour old cultures were streaked on the succinate medium amended with tertiary complex Chrome azurol S (CAS) / Fe^{3+} / hexadecyl trimethyl ammonium bromide as an indicator. The result was scored either positive or negative to this test, based on the colour change of the medium from blue to fluorescent yellow while no colour change was marked as absence of siderophore production.

Endoglucanase activity of *Methylobacterium* spp.

The epiphytic *Methylobacterium* spp. isolates were point inoculated using sterile tooth pick on Kim Wimpenny medium plates amended with and without D-glucose containing 1 % carboxymethyl cellulose. After 3 days of incubation at 30°C, the plates were flooded in 0.1 % congo red for 30 min. followed by 1 M NaCl as destaining solution for 10 min. Endoglucanase activity was determined by the appearance of clear yellow zones around the colonies (Reinhold-Hurek *et al.*, 1993).

Estimation and quantification of phytohormones

Cytokinin

Extraction of cytokinin

The epiphytic *Methylobacterium* spp. isolates were grown in AMS liquid medium for 7 days at 30°C in a rotary shaker at 250 rpm. The overgrown culture was centrifuged at 12,000 rpm for 30 min. and the supernatant was extracted twice with equal amount of n-butanol using separating funnel and the fractions were evaporated in petriplate at

room temperature. After evaporation, the cytokinin fractions were dissolved in 2 ml of 100 % absolute methanol (HPLC grade) followed by filter sterilization using 0.2 μ m membrane filter.

Quantification of cytokinin

Filter sterilized cytokinin fractions (20 μ l) were injected into high performance liquid chromatography (HPLC). Analysis was performed using a 10 μ m particle size reverse phase column (C18) with a solvent gradient of 30 per cent methanol in water at a flow rate of 1.5 ml min^{-1} with operating pressure of 300 psi. Quantification of cytokinin compounds in the sample was compared to the retention time with peak height of chemical grade benzyl adenine purine (BAP), kinetin, zeatin, zeatin riboside and trans zeatin HCl (Sigma grade). Substances were quantified by integrating the areas under the peaks using UV detector at 254 nm.

Auxin

Extraction of IAA

The amount of indole compounds in the culture liquid of *Methylobacterium* sp. isolates was determined in the logarithmic phase after removing cells by centrifugation at 10,000 x g for 30 min. Auxins were extracted with an equal volume of ethyl acetate from the culture filtrate acidified to pH of 2.5-3.0 with 0.1 N HCl. Then the extracts were dried, the residues were dissolved in 80 per cent methanol and the auxin present in the samples were separated by HPLC.

Quantification of IAA

The ethyl acetate fractions were evaporated and the sediment was dissolved in 3 ml absolute methanol (Ivanova *et al.*, 2001). The

membrane filtered extract of 10 µl was then injected into HPLC (Shimadzu, Japan). Analysis was performed using a 10 µm particle size reverse phase column (C18) with a solvent gradient of 30 per cent methanol in water at a flow rate of 1.5 ml min⁻¹ with the operating pressure of 300 psi. The standard solution of IAA was prepared in 10 to 50 µg concentrations, from that 10 µl was injected into HPLC. Substances were quantified by integrating the areas under the peaks using UV detector at 254 nm. The quantity of different indole compounds in the sample was calculated by comparison of peak height and retention time of respective standards.

Crop response studies

Selection and preparation of bacterial inoculum

Based on the phytohormones synthesis (zeatin, kinetin, BAP and IAA) three strains (Mt-Tm 8, 13 and 14) were selected to test its ability in improving the tomato crop growth and development. Selected isolates were grown in 25 ml AMS broth at 30°C for 10 days. The cell pellets were washed twice with sterile 0.03 M MgSO₄ and resuspended in phosphate buffer to an OD of 0.15 ± 0.02 at 600 nm to avoid the influence of growth medium, and used as inocula for seedling vigor assay under gnotobiotic conditions.

Surface sterilization of seeds

Tomato (variety PKM-1) seeds were obtained from Horticultural College, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Seeds were surface-sterilized as described by Senthilkumar *et al.*, (2009). Briefly seeds were surface sterilized with 70% ethanol for 1 min followed by 1% sodium hypochlorite (NaOCl) for 20 min, and washed thoroughly with sterile milli-Q water several times. From the last washings, one ml

of the aliquot was checked for the presence of bacteria by serial dilution technique and there was no bacterial growth indicating the complete surface sterilization of the seeds. Bacterial suspensions in sterile distilled water (10⁸cfu ml⁻¹) were used for seed imbibition; control seeds were treated with 0.03 M MgSO₄. The sterilized seeds were soaked in the inoculum for 15 minutes and then shade dried for 10- 15 minutes before sowing.

Seedling vigor assay

After surface sterilization, tomato seeds (20–30 seeds) were treated with respective bacterial isolates as mentioned above. Control seeds were incubated in sterile distilled water for the same period. Germination tests were carried out by the paper towel method. The germination paper was soaked in distilled water, 15–20 bacterially treated seeds and untreated seeds were placed on paper towels, rolled and wrapped with polythene to prevent drying, and incubated at above mentioned conditions. After 15 days, the towels were unrolled and the number of seeds that had germinated was counted. On the same day, seedling vigor was analyzed using the method of Abdul Baki and Anderson (1973). The lengths of roots and hypocotyls of all the individual seedlings were measured. The vigor index (VI) was calculated using the formula VI = (mean root length + mean hypocotyl length) x % germination. The experiment was repeated twice.

Greenhouse experiment

Study was conducted to assess the foliar application of *Methylobacterium* spp. on the yield of tomato in potted soil conditions. Three to five bacterized seeds were sown into plastic pots having diameter 45 cm and containing 10 kg sterile soil mixture having the physicochemical properties of pH 7.3, EC 1.3 dSm⁻¹, organic carbon 3.78 g kg⁻¹

available N 82 mg kg⁻¹, available P 9.4 mg kg⁻¹ and potassium 260mg kg⁻¹. The pots were pre irrigated to the field capacity before sowing. The soil compost used has a composition of soil: sand: compost in a ratio of 2:1:1. There were 8 treatments in all. Where, T1= un-inoculated control, T2= water spray, T3= Mt-Tm-8 foliar application @0.5%, T4= Mt-Tm-8 foliar application @1.0%, T5= Mt-Tm-13 foliar application @0.5%, T6= Mt-Tm-13 foliar application @1.0%, T7= Mt-Tm-14 foliar application @0.5%, T8= Mt-Tm-14 foliar application @1.0%. Fifteen days after sowing and during peak flowering stage foliar application of *Methylobacterium* spp. was done. The seed germination was noted on the 12th day of sowing. The pots were irrigated routinely. The experiment was conducted in a completely randomized block design and each treatment was replicated five times. Seedling growth, phenology, were recorded. Two plants from each pot were randomly selected for recording the data. The whole plant dry weight (g plant⁻¹), plant height (cm), number of leaves per plant, number of fruits per plant, yield per plant (g), average fruit weight per plant, chlorophyll and protein were observed. Relative water content and proline content was measured as given in Quilambo, (2014).

Data analysis

The data were measured and subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS, version 10.0). Where there was statistical significance (P = 0.05), the mean values were further separated using the Duncan's multiple range test (DMRT).

Results and Discussion

Most microbes inhabiting plant-related niches have neutral or beneficial roles in plant health and development (Philipot *et al.*, 2013).

Microorganisms can affect agricultural productivity, for instance by assisting and controlling nutrient availability/acquisition and promoting stress tolerance (Kavamura *et al.*, 2013). Most of the research on this topic has focused on the functional roles of single microbial groups (*e.g.*, specific species or organisms from the same genera) associated with plants, mostly because of methodological limitations to assess non-culturable microbial groups (Andreote *et al.*, 2009). Examples of these inferences are related to specific microbial groups able to promote plant growth, such as nitrogen-fixing bacteria (Raymond *et al.*, 2004) and mycorrhiza-forming fungi (Chagnon *et al.*, 2013). The cascade of events that occurs after a bacterial cell recognizes host plant results in major changes in cellular metabolism, including the accumulation of several secondary metabolites (Hahlbrock *et al.*, 2003). Such physiological changes can modulate the growth and development of the plant. However, considering their complexity, these mechanisms and networks are still far from being elucidated. Therefore, different studies have been conducted with the aim of uncovering these mechanisms.

Plant growth stimulation by epiphytic bacteria is largely due to phytohormone production, and several studies have been reported the interaction of *Methylobacterium* species with different plant species by regulating phytohormone production (Schauer and Kutschera, 2011). *Methylobacterium* strains have been reported to produce phytohormones such as cytokinins and auxins (Pirttila *et al.*, 2005), which promote cell division and elongation, respectively. A total of 32 endophytic bacterial colonies were characterized for colony morphology, cell shape, Gram reaction and pigment production followed by biochemical characters (data not shown). All the *Methylobacterium* isolates were rod shaped and Gram negative. The

colony morphology varied from circular, blend, raised, convex, and low convex and pulvinate. The isolates also exhibited varied colony colours *viz.*, whitish pink, light pink, orangish pink, pink and dark pink (data not shown). In static culture, all the selected strains formed a pink surface ring and/or pellicle, this showed that the strains tested were strict aerobes and were tentatively identified as *Methylobacterium* sp. All the strains failed to degrade or hydrolyze casein, starch, gelatine and cellulose. None of the isolates possessed lipolytic activity. All the strains showed positive for catalase, oxidase and urease activity. The indole production, methyl red and Voges Proueskauer tests were negative in all the strains tested. The epiphytic isolates did not reduce nitrate to nitrite and absence of denitrification and H₂S production was also noticed. In addition, they can form biofilms (Rossetto *et al.*, 2011) and use methylotrophic metabolism as an adaptive advantage during plant host colonization (Sy *et al.*, 2005). Members of the *Methylobacterium* genus occupy different habitats due to their great phenotypic plasticity, including soil, water, leaf surfaces, nodules, grains, and air (Madhaiyan *et al.*, 2012).

Assessing the PGPR traits

During interactions with plants, *M. nodulans* and *M. radiotolerans* have been reported to be involved in nitrogen fixation and nodule formation (Sy *et al.*, 2001), while other *Methylobacterium* species may be related to phytohormone production (Meena, 2012) or interacting with plant pathogens (Lacava *et al.*, 2004), promoting plant growth (Madhaiyan *et al.*, 2006) and inducing higher photosynthetic activity but few reports were related to PGP epiphytic bacteria from tomato plant tissues (Shen *et al.*, 2012). Five isolates were randomly selected and tested for its abilities to promote plant growth. Almost all

selected *Methylobacterium* isolates exhibited more than three plant growth promoting traits. These results showed that the microorganisms isolated from similar environment showed similar plant growth promoting character. This can be explained as the regional characteristics and environmental selectivity (Sheng *et al.*, 2011). Firstly, their ability to produce cytokinins and indole-3-acetic acid (IAA) were tested. All five *Methylobacterium* isolates produced significant amounts of cytokinins like zeatin, kinetin, benzyl amino purine (BAP) and IAA except Mt-Tm-9 and Mt-Tm-10 did not synthesis BAP and kinetin respectively (Table 1). The biosynthesis of zeatin was significantly higher for Mt-TM-13 which produced 4.94 µg ml⁻¹, followed by Kinetin and BAP. Several reports indicated that even strains belonging to the same genus produced different amounts of IAA, because it was affected by culture conditions, growth stage or substrates (Ahmad *et al.*, 2008). It is a well-established fact that IAA regulates several cellular and plant growth processes (Lambrecht *et al.*, 2000). Similarly, the bacterial isolates obtained from Maize (Montañez *et al.*, 2012), Tomato (Rashid *et al.*, 2012) and Wheat (Durán *et al.*, 2014) were able to produce IAA. Growth on N – free medium, ACC deaminase activity, production of siderophore on a R2A-CAS medium were assayed. Variable results were obtained among the five isolates. All isolates formed a halo around the colonies on this medium and were considered to be siderophore positive. Production of siderophore is one of the traits for the bacteria to compete with other bacteria to colonize plant roots and leaf as well as inhibit the pathogens (Ahmad *et al.*, 2008). Loaces *et al.*, (2011) showed that the 11 rice leaf epiphytic isolates were able to produce siderophore, the isolates were belonging to *Pantoea* and *Pseudomonas* genera. The N₂-fixing ability of bacterial epiphytes was also screened on a N₂-free media. Out of selected five isolates, only

two isolates (Mt-Tm-10 and 13) showed the capacity to grow in nitrogen-free conditions confirming the significance of potential N₂-fixing ability for endophytic bacteria in the host plant. Except Mt-Tm-10 all isolates showed the endoglucanase activity (Table 2).

Plant response to *Methylobacterium* spp. isolates application

Seed Vigor and Seedling Growth

The purposes of introducing bio-inoculant modulating seed microbiomes is to achieve improvement of a desired agricultural trait such as growth enhancement of the offspring plant. Treatment of seed with beneficial micro-organisms including fungi and bacteria (species of *Trichoderma*, *Pseudomonas*, *Bacillus*, *Rhizobia* etc.) ameliorates a wide variety of biotic, abiotic, and physiological stresses to seed and seedlings (Mastouri *et al.*, 2010). Inoculation of seeds with such biological agents in combination with priming (Bioprimering) potentially able to promote

rapid and more uniform seed germination and plants growth (Moeinzadeh *et al.*, 2010) and in several cases, has been reported to enhance and stabilize the efficacy of biological agents (Warren and Bennett, 1999).Seed imbibition with different types of *Methylobacterium* isolates showed significant variation in the vigor index and germination (Table 3). Tomato seeds imbibed with *Methylobacterium* isolates at two different concentrations (0.5% and 1.0%) had induced the earliness in radical emergence on 4th day after treatment with more than 90% seeds showed radical protusion and 100 % germination was attained on 10 days after sowing with Mt-TM-8, and 13 strains. In a report by Long *et al.*, (2008), a *Solanum nigrum* seedling vigour assay was carried out to screen the epiphytic bacterial isolates for their PGP ability such as on seed germination, root and hypocotyl growth and 37 of 77 isolates increased seedling vigour and 22 isolates significantly enhanced seed germination up to 100 % compared to untreated controls.

Table.1 Screening of epiphytic *Methylobacterium* spp. for plant growth hormones production

Strains	Zeatin (µg ml ⁻¹)	Kinetin (µg ml ⁻¹)	BAP (µg ml ⁻¹)	IAA (µg ml ⁻¹)
MtTm8	0.12 ±0.00	10.1 ±0.51	0.16 ±0.01	102.3 ±1.20
Mt TM 9	0.68 ±0.17	1.61 ±0.06	-	86.9 ±1.20
MtTm10	0.90 ±0.11	-	0.12 ±0.00	29.8 ±0.65
MtTm13	4.94 ±0.16	6.03 ±0.01	0.14 ±0.01	88.1 ±1.16
MtTm14	0.65 ±0.02	1.04 ±0.04	0.11 ±0.00	7.64 ±0.11

Table.2 Plant growth promoting activities of epiphytic *Methylobacterium* spp. isolated from phyllosphere region of tomato

Strains	Growth in N free medium	ACC deaminase	Siderophore production	Endoglucanase activity (Glu ⁺)
MtTm8	-	+	+	+
Mt TM 9	-	-	+	+
MtTm10	+	+	+	-
MtTm13	+	+	+	+
MtTm14	-	+	+	+

+ indicates the presence of growth; - indicates the absence of growth; Glu⁺ - glucose and N - Nitrogen

Table.3 Effect of *Methylobacterium* spp. on tomato seed germination and vigor index

Treatments	Seedling parameters								
	Percentage of radical protusion	Germination % on 4 th day	Germination % on 10 th day	Growth on 10 th day (Av. 25 seedlings / replication) (cm plant ⁻¹)		Days for maximum germination	Vigor index	Biomass on 10 th day (Av. 25 seedlings/ replication) (mg plant ⁻¹)	
				Shoot length	Root length			Fresh wt	Dry wt.
Uninoculated control	28.50	52.5	65	5.5 (±0.29)	4.0 (±0.29)	9	617.5	379.66 (±21.62)	75.00 (±6.69)
Water	64.28	79.16	86.6	6.0 (±0.17)	4.0 (±0.00)	7	866	414.33 (±5.46)	113.33 (±5.24)
0.5 % MtTm8	76.19	89.16	100	6.33 (± 0.17)	4.16 (±0.17)	5	1049	430.66 (±4.63)	82.33 (±2.08)
1.0 % MtTm8	90.47	98.3	100	6.66 (±0.33)	4.5 (±0.00)	5	1116	465.66 (±11.41)	149.66 (±4.70)
0.5 % MtTm13 spray	78.57	96.83	100	6.5 (±0.50)	4.33 (±0.33)	5	1083	466.33 (± 5.90)	141.33 (±6.12)
1.0 % Mt-Tm-13 spray	95.23	99.16	100	6.83 (±0.17)	4.83 (±0.17)	4	1166	473.33 (±3.38)	149.66 (±7.06)
0.5 % Mt-Tm-14 spray	78.57	93.33	95.83	5.83 (±0.17)	4.16 (±0.17)	6	957.34	465.66 (±3.38)	149.66 (±4.33)
1.0 % Mt-Tm-14 spray	78.57	92.5	100	6.0 (±0.00)	4.33 (±0.17)	6	1043	436.66 (±6.94)	126.33 (±5.04)
CD	4.28	3.42	5.08	1.3	0.2	0.9	3.1	3.9	2.0
SEd	2.21	1.6	2.39	0.6	0.08	0.51	1.7	2.06	1.12

Table.4 Effect of *Methylobacterium* spp. on tomato plant growth and yield

Treatments	Plant parameters						
	Shoot length at harvest (cm plant ⁻¹)	Root length at harvest (cm plant ⁻¹)	Average number of fruits (No.plant ⁻¹)	Average Weight of individual fruits / plant (g)	Average yield/ plant (g)	Dry wt of plant (g plant ⁻¹)	Population of <i>Methylobacterium</i> at the time of flowering (cfu g ⁻¹ fresh weight of leaf)
Uninoculated control	79.99 (±0.19)	55.73 (±0.48)	6.7 (±0.30)	29.31 (±0.64)	178.92 (±3.19)	46.07 (±1.16)	22x10 ⁴
Water spray	79.66 (±0.38)	54.00 (±2.23)	6.7 (± 0.10)	29.47 (±0.93)	193.66 (±1.26)	47.05 (±0.98)	38x10 ⁵
0.5 % MfTm8 spray	80.55 (±2.54)	58.44 (±0.44)	7.8 (±0.30)	31.64 (±0.65)	205.94 (±0.53)	48.46 (±0.08)	49x10 ⁶
1.0 % MfTm8 spray	92.77 (±1.06)	61.60 (±1.77)	8.2 (±0.10)	32.79 (±1.19)	238.94 (±1.77)	52.86 (±2.48)	36x10 ⁶
0.5 % MfTm13 spray	90.66 (±0.38)	60.28 (±1.14)	8.0 (±0.33)	30.61 (±1.98)	236.40 (±2.91)	49.13 (±0.26)	26x10 ⁶
1.0 % MfTm13 spray	97.55 (±0.29)	62.68 (±1.28)	8.8 (±0.30)	33.72 (±1.82)	257.62 (±1.76)	53.85 (±1.92)	68x10 ⁶
0.5 % MfTm14 spray	85.44 (±0.78)	58.66 (±1.94)	7.7 (±0.57)	31.11 (±1.43)	222.36 (±3.24)	48.07 (±0.80)	45x10 ⁶
1.0 % MfTm14 spray	88.99 (±0.70)	60.97 (±1.00)	8.0 (±0.38)	32.18 (±1.14)	237.95 (±14.15)	49.52 (±1.14)	46x10 ⁶
CD	1.8	1.24	0.51	0.67	5.18	1.1	
SEd	0.86	0.7	0.32	0,29	2.74	0.61	

Fig.1 PPFM spray on tomato leaf relative water content and proline content

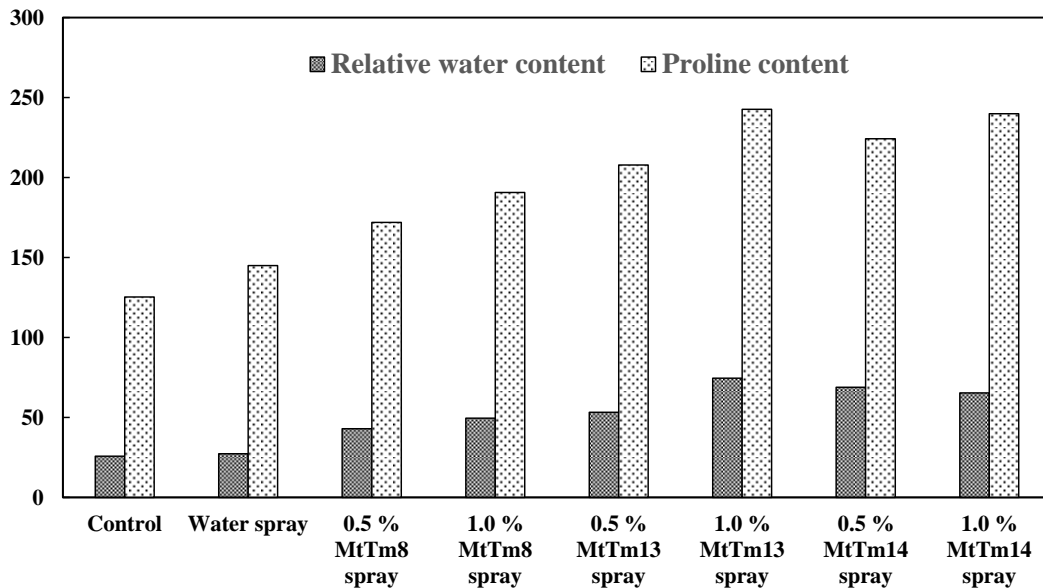
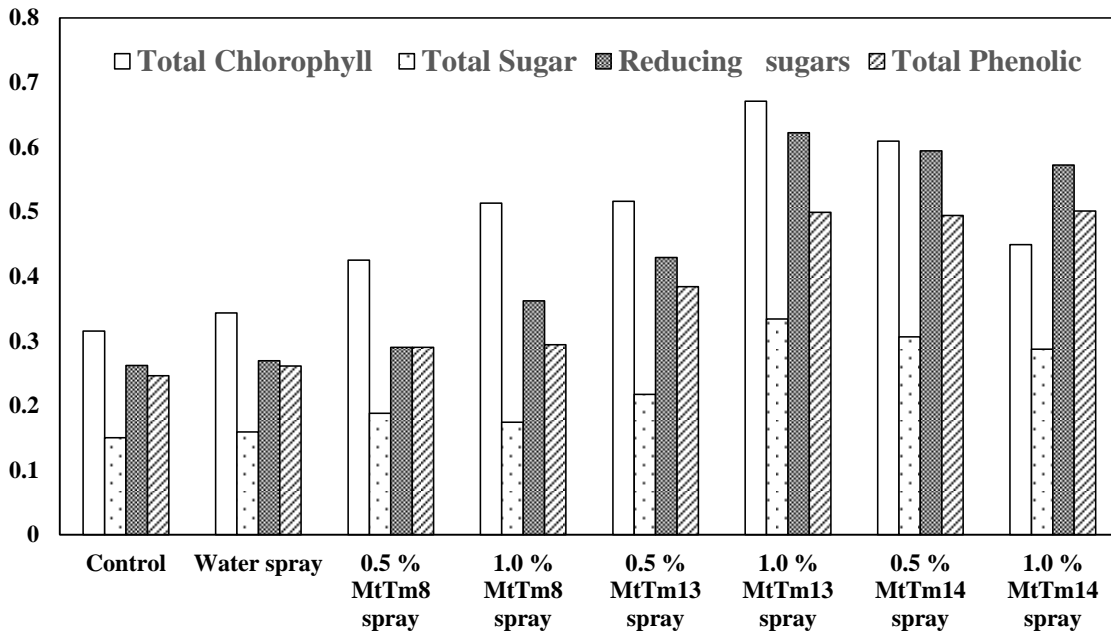


Fig.2 Effect of PPFM spray on physiological parameters of tomato plant



Ten days after seedling growth, treated seeds showed increased shoot length and root length. The maximum shoot and root length of 6.83 cm plant⁻¹ and 4.83 cm plant⁻¹ was observed in 1.0% treated seeds with Mt-Tm-

13. From the *Methylobacterium* treated seedlings, an average of 21% and 20% increase in fresh weight and dry weight was observed over the uninoculated control seedlings (Table 3). The bioinoculant

Methylobacterium treated seedlings also showed better vigor index over the control treatments.

Effect of *Methylobacterium* application on tomato plant growth and yield

Under the green house condition, the foliar application of *Methylobacterium* had significantly improved the overall performance of the tomato plant growth and yield over uninoculated plants (Table 4). Effect of foliar application of *Methylobacterium* isolate Mt-Tm-13 at 1% concentration showed significant increase in plant height and yield followed by 1% foliar spray of Mt-Tm-8. The vast majority of microbes in the phyllosphere exerts no negative influence on plant growth and development and, in fact, often confer a positive effect (Bulgarelli *et al.*, 2013). The significant difference in plant height at different stages were noticed. The maximum plant height of 97.55 cm plant⁻¹ and root length of 62.68 cm plant⁻¹ was observed in Mt-Tm-13 treated plants with 1% concentration. The increased growth could be attributed to the growth hormones like IAA and cytokinin produced by *Methylobacterium* which stimulated cell division, cell enlargement and influence root morphology. This in return, would have improved assimilation of nutrients. Increase in yield component may also be attributed due to increase in fruit weight as a result of improvement in adequate plant nutrition uptake. As already mentioned before, PPFMs are frequently found on leaf surfaces. Abanda-Nkpwatt *et al.*, (2006) reported about a *Methylobacterium extorquens* strain that promotes growth of various plant seedlings. Cytokinins stimulate a plant's cell division, vascular cambium sensitivity, and vascular differentiation and induce the proliferation of root hairs, but inhibit lateral root formation and primary root elongation. Liu *et al.*, (2013)

reported that the oriental Thuja seedlings inoculated with cytokinin-producing *Bacillus subtilis* strains were more resistance to stress.

Significant variation was recorded among different treatment combinations for yield attributing characters. Among the different *Methylobacterium* isolates, plants treated with Mt-Tm-13 had maximum number of fruits (8.8 number plant⁻¹). The other isolates also had better yield response when compared to control. The increase in number of fruit per plant is due to increase in individual weight of the fruit which in turn has influenced the overall the yield of the crop.

Similarly, relative water content and leaf proline content were found to be higher in the plants treated with *Methylobacterium* isolate Mt-Tm-13 (Figure 1). Physiological parameters such as chlorophyll and sugar content were higher in Mt-TM-13 isolate applied plants (Figure 2).

In this study, the *Methylobacterium* spp. strains enhanced the plant growth parameters of tomato under *in vitro* condition by secreting significant amount of cytokinin (zeatin) in their culture media as is evident from HPLC analysis which ranged from 0.11 µg ml⁻¹ to 4.94 µg ml⁻¹ and this was earlier reported for *Bacillus subtilis* (1.2 mg. l⁻¹) (Arkhipova *et al.*, 2007), *Azotobacter vinelandii* (0.75 µg l⁻¹). In addition to zeatin, *Methylobacterium* isolates also produce significant amounts of kinetin and benzyl adenine purine (BAP). The kinetin and BAP phytohormones produced by *Methylobacterium* sp. is an evidence in improving the plant growth parameters but their exact mechanism with respective gene identification is further needed. The level of cytokinin production by the bacterial strains showed positive effect on tomato seedling growth. This may be contributed by several authors that, methylobacteria can also

produce other phytohormones rather than zeatin and this assumption was proven in the present study. In most cases, these phytohormones are believed to be changing their assimilate partitioning patterns in plant system which results in bigger and more branched root with greater surface area (Vessey, 2003). Evidence for the role of phytohormone synthesis of auxin, cytokinin and also ACC deaminase (Madhaiyan *et al.*, 2006) by *Methylobacterium* sp. and its plant growth promoting activity from the studies of Holland (1997) showed this genera can be exploited for its endophytic association and diazotrophic property (Sy *et al.*, 2001).

Current study showed the positive impacts of *Methylobacterium* on growth and yield of tomato plant compared to control. In addition to foliar spray introduction of *Methylobacterium* through tomato seeds before planting is easy and cost effective method to improvement seedling and plant growth efficiency. These results proved that plant growth regulators produced by *Methylobacterium* species could also play a critical role in plant growth promotion.

More understanding is currently provided through mechanism-based studies, and using ecological, genetic and ‘-omics’ approaches. Despite this effort currently carried out by an increasing number of research teams, several questions and hypothesis should still be put on the table and contrasted (Stéphane *et al.*, 2016). Studies on the role of PGP traits on plant bacterial interaction may reveal how this growth promoting traits producing bacteria helps in plant growth. Several studies related to epiphytes are, moreover, mostly biased by experimental models evaluated under gnotobiotic conditions, that is, far away from natural conditions. We need to move beyond and to analyze how the whole plant and its associated phyllosphere microbiota interact for symbiotic association.

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