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Comparative Analysis of two *Methylobacterium* spp. for Mitigating Moisture Stress Induced by PEG in Early Growth Stages of Tomato

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A B S T R A C T

Tomato (*Solanum lycopersicum*) is widely cultivated vegetable crop and processed throughout the world which is highly susceptible to moisture stress. In this study *Methylobacterium* spp. *viz., M. aminovorans* and *M. thiocyanatum* were used to increase the moisture stress tolerance. Artificial moisture stress was induced to tomato seeds using Polyethylene Glycol (PEG) with concentration ranged from 0 to -3bars.The results showed that highest growth parameters were exhibited in tomato seedlings treated with *M.thiocyanatum*, where it exhibited maximum germination percentage at 0 bar (42.7%), vigor index at 0 bar (170.8), shoot length at -3bar (2.7 cm),root and seedling length at -2 bar (4.1cm, 5.1cm) followed by *M.aminovorans*, which showed germination percentage of 40% at (-1.5 bar), vigor index of 131.2 at (-1 bar), root, shoot and seedling length of 2.9cm, 1.9cm, 4.8cm at (-3 bar) respectively. Compared to treated seeds, the un-inoculated control showed lowest germination percentage at 0 bar (17.2%), vigor index (48.3), root, shoot and seedling length (1.7cm, 1.1cm, 2.2cm). Root hair deformation was observed in both treated seedlings which are grown under aseptic condition. In which, *M. aminovorans* performed well when compared to *M. thiocyanatum*. The activity of antioxidant enzymes catalase (CAT), peroxidase (PER), proline, glycine betaine (GB) and superoxide dismutase (SOD) were enhanced by *M. aminovorans* at -1.5 bars & *M. thiocyanatum* at -2 bars which have the ability to protect the plant under moisture stress conditions. Thus, *Methylobacterium* has the ability to mitigate drought stress and increase the drought tolerance of tomato crop.

Introduction

Tomato (*Solanum lycopersicum*) is the most cultivated vegetable crop in the world. The global production of tomato was around 164 million tonnes, which covers an area of 4.8 million hectares with a seventh place rank in productivity (FAOSTAT, 2013; Luna *et al.*, 2016). Presently global productivity of tomato was facing the difficulties of biotic and abiotic stress. While tomato is a day neutral plant and which is highly susceptible to high temperature and drought. Drought is the most important environmental stresses in agriculture which directly reduces the crop growth and yield by limiting the global productivity.

Polyethylene Glycol (PEG - 6000) induces water stress artificially in plants and existence in the form of viscous liquid to solid wax (Larher *et al.,* 1993). It reduces the cell water potential by inducing osmotic stress (Govindaraj *et al.,* 2010). During the process of photosynthesis the induction of osmotic stress in plants causes changes at the cellular level which affects pigments present inside the chlorophyll (chlorophyll-a & b) which decreases the rate of photosynthesis, photosystems, and the transport mechanism of electron and pathways of CO_2 reduction. Dodd and Donovan, (1999) investigated that drought stress also induced the inhibition of germination (Sidari *et al.,* 2008).

Shukla *et al.,* (2012) reported that diverse tolerance mechanisms occur due to changes in physiological and biochemical process to withstand the drought stress condition. Recent studies had reported that plant-associated bacteria can help more effectively to withstand abiotic stresses (Chauhan *et al.,* 2015). Vadharajula *et al.,* (2011) studied the role of rhizosphere bacteria in stress tolerance but few studies focused on phyllosphere bacteria which involves in improvement of plant environmental condition by suppressing the activity of biotic and abiotic stress. Mostly study on phyllosphere bacteria had focused on individual bacteria isolates using culture-dependent methods in terms of their role in pathogen suppression in plant defense (Delmotte *et al.,* 2009) and in plant growth promotion (PGP) (Papen *et al.,* 2002) or through the production of 1-aminocyclopropane-1carboxylate (ACC) deaminase. Phyllosphere colonization by *Methylobacterium* has been reported to produce phytohormones such as cytokinin and auxins (Madhaiyan *et al.,* 2005) and stress response enzyme ACC deaminase (Chinnadurai *et al.,* 2009). *Methylobacterium* influences seed germination and plant growth by cytokinin and auxin synthesis (Omer *et al.,* 2004; Tani *et al.,* 2012; Eevers *et al.,* 2015), nitrogen fixation (Sy *et al.,* 2001; Madhaiyan *et al.,* 2014) and plant protection (Ardanov *et al.,* 2012; Madhaiyan *et al.,* 2006, Yim *et al.,* 2014). Exogenous use of PPFM has some advantages in alleviating the adverse effects of drought stress and also improves the germination, production, development, quality and yield of crops (Hayat *et al*., 2010).

The aim of the present study was to investigate the comparative effect of two species of *Methylobacterium* and generated osmotic stress by different concentration of PEG on seedling characters, root hair deformation assay and biochemical parameters of tomato.

Materials and Methods

Bacterial strains, culture conditions and plant material

The *Methylobacterium* strains: *M. aminovorans* (MtTm13) (KC568445), *M. thiocyanatum* (DSM11490^T) were obtained from Department of Agricultural Microbiology, Agricultural College and

Research Institute, Madurai, Tamil Nadu. *Methylobacterium* strain were cultured in Ammonium Mineral Salt (AMS) broth with 0.5% methanol and these bacterial cells were incubated for 10 days to get a maximum cell population of 10^9 cfu ml^{-1}.The tomato seeds variety PKM1 were obtained from Department of Vegetable Crops, Horticultural College and Research Institute, Periyakulam, Tamil Nadu.

Mechanism of inducing Drought stress

The experiments were performed in petridish under *In vitro* condition. The tomato seeds are surface sterilized with 0.02% $HgCl_2$ for 5 minutes followed by 70% ethanol for 1 min followed by two subsequent rinses in sterile distilled water. Twenty five seeds per treatment were soaked in respective *Methylobacterium* spp. for 4 hours in liquid cultures (10^9 colony-forming units [CFU] /ml)). The treatments are (T_1) un-inoculated seeds (control) treated with sterile distilled water; (T_2) seeds inoculated with *M.aminovorans;* (T_3) seeds inoculated with *M.thiocyanatum*. The seeds were drained in laminar air flow cabinet for 10 minutes. Then the treated seeds were placed in a petridish containing filter paper, untreated seeds were taken as control and kept in an incubator.

After 12 hours respective concentration of PEG solution (0 to -3bars) was applied to each treatment. Number of seeds germinated was manually counted on each day up to 15 days and the seed germination characters were considered based on the emergence of radicle and plumule. The experiment was performed in five replicates for each treatment and statistical analyses were carried out and compared the growth of both treated and untreated seeds added with PEG. On 15^{th} day, speed of seed germination (Maguire 1962), germination percentage, vigor index (Abdul Baki and Anderson, 1973), root and shoot length was observed

Root hair deformation assay

The surface sterilized tomato seeds as mentioned in above process were treated with *Methylobacterium* strains and placed in moistened filter paper in a petridish and was incubated at 25^0C. After 12 hours of incubation respective PEG solution (0 to -3bars) were added to each treatment. After 5-7 days the tomato seedlings showed abundant root hair growth in lateral root. All the root hairs were observed under microscope by mounting one root per presterilized slide where the entire root was observed to be not deformed. Thus, *Methylobacterium* strains were added to the seedlings which are kept for a period of incubation, and then the roots of treated seedlings were taken and stained by aniline blue/ lactophenol stain (White *et al.,* 2014) in which root hairs deformation were observed. The experiment was performed in five replicates for each treatment. The treated and untreated seedling root hairs were observed using Bright field microscope (M/s. Olympus, Japan) at 40x magnification. Number of root hairs were counted, morphological feature of primary infected root and root hair length, root hair breadth was observed.

Enzyme extraction and assays

Fresh tissue (l00mg) was homogenized in an ice cold mortar using 50 mM potassium phosphate buffer pH 7.0. The samples were centrifuged at 11,000 rpm for 25 min at 4°C and the supernatants were used to determine the soluble protein content for SOD, CAT, POX, Glycine betaine and Proline activities. SOD, CAT, POX, Glycine betaine and Proline enzyme activities were determined according to methods described by Dhindsa *et al.,* (1981), Chaparro-Giraldo *et al.,* (2000), Hammerschmidt *et al.,* (1982), Greive and Grattan (1983), and Bates *et al.,* (1973), respectively.

Statistical Analysis

The experiments were conducted in completely randomized block design. The results presented are the means of three replicates. The data obtained were analyzed by a one factorial analysis of variance, with substrate as experimental factor using AGRES software as per described by Panse and Sukhatme (1978).Sample variability was estimated by standard deviation of the mean. Analysis of variance was conducted on the data at CD (P=0.05).

Results and Discussion

Effect of PEG -6000 (0 to -3bars) induced osmotic stress on growth parameters

Speed of germination

Methylobacterium spp. influenced the speed of seed germination and results were presented in table1. The result showed that un-inoculated control at 0 bar showed earlier speed of germination (1.91) was due to their ability to absorb water in normal condition. Induced drought stress in the seeds by adding PEG reduced the speed of germination. The induced moisture stress by PEG decreased the speed of germination at -1 bar (1.21) followed by 0.86 at (0.5 bar), 0.60 at (-1.5 bar). In the *M. aminovorans* treated seeds added with PEG showed faster germination of seedlings at -1.5 bar (3.2) followed by -1 bar (3.0), whereas the *M. aminovorans* treated seed with PEG has the 2.3 at 0 bars. In the *M. thiocyanatum* treated seeds, maximum speed of seed germination was observed at treatment without PEG (0 bar) with other treatments showed decline in germination speed. When comparing other treatments, speed of germination declined as the concentration of PEG increased irrespective of the treatments. In the un-inoculated treated seeds with PEG alone the germination was arrested beyond -1.5 bars hence results are depicted from 0 to -1.5 bars presented in table 1. Similar results were found in Febri Doni *et al.,* (2014) rice seeds treated with *Trichoderma* sp., revealed speed of seed germination were significantly greater than un-inoculated control. Raja *et al.,* (2018) reported that the seed treatment with microbial cultures increased the speed of germination in paddy. The effect of PGPR inoculation was significantly enhancing the speed of germination on *Crataegus pseudoheterophylla* (Fatemeh *et al.,* 2014). Among the two *Methylobacterium* species the *M. aminovorans* treated seeds added with PEG showed faster germination of seedlings at -1.5 bar (3.2). The two species used in this study had confirmed the induced moisture stress tolerance to the treated seeds with varying result.

Germination rate (%)

Number of germinated seeds of the tomato variety PKM 1as affected by PEG treatment and the results showed that un-inoculated control had germination rate after 15 days at 0 bar was 17.2% while the least germinated rate were exhibited at -1 bar (10.5%), -0.5 bar (7.9%) and -1.5 bar (4%). In the *M. aminovorans* treatment had higher germinated rate at -1.5 bar (40%) followed by -1 bar (32%), while the least germinated rate were exhibited at -3bar (18.5%). Treated seeds of *M. thiocyanatum* showed earlier germinated percentage at 0 bar without PEG (42.7%) with other treatments showed decline in germination percentage given in table 1. Similar results of improved seed germination and also plant growth promotion on tomato under drought condition was reported by Chandrasekaran *et al.,* (2017). Madhaiyan *et al.,* (2006) demonstrated the PGP *Methylobacterium* containing ACC deaminase in the roots of canola (*Brassica campestris*) and there by reduced the concentration of ACC and ethylene level due to the activity of bacterial ACCd (ACC deaminase).

Methylobacterium inoculation increased the IAA concentrations of the plants resulting in increased ACS (ACC synthase) activity.

Saikia *et al.,* (2018) reported that the ACC deaminase-containing methylotrophic bacteria could reduce a significant portion of the plant damage by breaking down the ethylene precursor into ammonia and α-ketobutyrate. ACC deaminase produced by bacteria was well proven to mitigate stress in various plants.

Jorge *et al.,* (2019) suggested that the cytokinin plays an important role in host plant metabolism, including stimulation of growth, increased level of photosynthesis and chlorophyll, as well as nutrient allocation and effective water management under drought condition. Kumar *et al.,* (2016) reported that inoculation of *Bacillus amyloliqufaciens* and *Pseudomonas putida* enhanced the germination percentage in chickpea under stressed condition due to bacteria had a higher potential to mitigate the stress. Ryu *et al.,* (2006) demonstrated that germination percentage of the *Methylobacterium* sp., treated tomato and red pepper seeds were comparatively greater when compared with un-inoculated control.

In addition, Meena *et al.,* (2011) reported that enhanced seed germination of wheat (*Triticum aestivum*) with highest values of 98.3% observed using *Methylobacterium* sp., Influence of *Methylobacterium* on seed imbibition to improving germination percentage of barnyard millet under dry land condition was reported by Poorniammal *et al.,* (2020). When comparing other treatments, germinated rate declined as the concentration of PEG increased irrespective of the treatments. Among the two *Methylobacterium* species the *M. aminovorans* treated seeds added with PEG showed faster germination of seedlings at -1.5 bar (40%) given in table 1.

Root, shoot and seedling length

Root, shoot and seedling length were assessed at the end of germination test. The result showed that un-inoculated control had the highest values of root, shoot and seedling length at 0 bar (1.7cm, 1.1cm, and 2.2 cm).

In the induced moisture stress by PEG the least values were observed at -0.5 bar (1.3cm, 0.6cm, 1.9cm) followed by -1 bar (1.4cm, 0.6cm, 2.0cm), -1.5 bar (0.7cm, 1.0cm, 1.8cm). The treated seeds of *M. aminovorans* showed maximum values of root, shoot and seedling length were observed at -3 bar (2.9cm, 1.9cm, 4.8cm) followed by -1 bar (2.7cm, 1.4cm, 4.1cm) while the least values were recorded at -0.5 bar (1.2cm, 0.6cm, 1.7cm) presented in table 1. In the *M. thiocyanatum* showed maximum values of shoot length at -3 bars, root and seedling length at -2 bar (2.7cm, 4.1cm 5.1cm).

As per the result of Ghosh *et al.,* (2003) the *Bacillus* sp., inoculated in rice seeds showed higher root and shoot length than control thus, the bacteria increased the uptake of nutrients and water by plant, by increasing the root length. *Methylobacterium spp.* significantly increased the seedling height of red pepper and tomato (Madhaiyan *et al.,* 2006; Ryu *et al.,* 2006).

Priya *et al.,* (2019) reported that the effect of single strain of *M. radiotolerans* in groundnut significantly increase the root and shoot length when compared to un-inoculated control. Inoculation of methylotrophic bacteria significantly enhanced the plant parameters of root and shoot length on groundnut (Krishnamoorthy *et al.,* 2019). Among the two *Methylobacterium* species the *M. thiocyanatum* treated seeds added with PEG showed maximum shoot length at -3 bar, root and seedling length at -2 bar (2.7cm,4.1cm 5.1cm) of seedlings presented in Table 1.

Vigor index

The vigor index of un-inoculated control showed highest value at 0 bar (48.3).The induced moisture stress by PEG the least values were recorded at1.5 bar (7.2), followed by -0.5 bar (14.9), 1 bar (21.2).In the *M. aminovorans* showed maximum vigor index at -1 bar (131.2) followed by -1.5 bar (116.5) while the least values were recorded at -0.5 bar (43.2). The treated seeds of *M. thiocyanatum* showed maximum vigor index at 0 bar (170.8) with other treatments showed decline in vigor index given in table 1. Similar results are in support of the findings of Kumar *et al.,* (2016) reported that the inoculation of *Bacillus amyloliqufaciens* and *Pseudomonas putida* enhanced the vigor index in chickpea under stressed condition and increasing potential to mitigate the stress. Senthil kumar and Krishnamoorthy *et al.,* (2017) reported the influence of *Methylobacterium* inoculation on increasing the seed germination and vigor index of tomato over un-inoculated control. In addition to this Poorniammal *et al.,* (2020) conducted a field experiment under dry land condition to study the effect of *Methylobacterium* on seed imbibition to improving vigor index of barnyard millet. When comparing other treatments, vigor index declined as the concentration of PEG increased irrespective of the treatments. Among the two *Methylobacterium* species the *M.aminovorans* treated seeds added with PEG showed maximum vigor index of seedlings at -1 bar (131.2) presented in table 1.

Root hair deformation

The root hair deformation assay were carry out in tomato seedlings give away existence of deformed root hair structures in Methylobacterial treatments. A tiny lateral roots containing the site of deformation in root hairs was observed, (Clavijo *et al.,* 2015) in which the deformation of root hair was triggered in a limited area of root zone, whereas it leads to arrest the enlargement of root hairs. Treated with *Methylobacterium* culture resulted in deformed root hairs with bulging at the root tip that were also observed in the tomato seedlings of present study. The assessment of root segment under bright field microscope exhibit hemispherical bulbous structures and resembles thick short root hair that could not be observed in un-inoculated tomato seedlings and the similar result was showed in the rice crop (Senthil kumar *et al.,* 2009) (Fig 1.). For inoculated seedlings, the most striking results of bacterial inoculation on root growth were a significant increase in the production of root hairs. For plants inoculated with the rhizospheric strains *Ensifer sp., Chryseobacterium* and all endophytes inoculated seedlings increases of root hair formation were observed (Abba Mondi *et al.,* 2016). In the *Methylobacterium* treated seedlings observed the number of root hairs increased in a 1cm section from the starting point of the root to the differentiation zone as compared with control seedlings. The treated *M. aminovorans* exhibit maximum number of root hairs (450) followed by *M. thiocyanatum* (441) as compared to un-inoculated control (430) presented in table 2. Similar results were found in the maximum number of root hairs were formed in treated *Methylobacterium* strains as compared to control (Krishnamoorthy *et al.,* 2018). Infected root hairs of *M. aminovorans* and *M. thiocyanatum* appeared like root tip bulging and thick short root hair followed by un-inoculated control resembles like long and slender root hairs. The maximum number of infected root hairs in *M. aminovorans* (255) followed by *M. thiocyanatum* (223) and an un-inoculated control showed no root hair infection. In un-inoculated control showed greater root hair length (104.4µm) as compared to treated *M. aminovorans* (80.2µm) and *M. thiocyanatum* (72.4µm).

Table.1 Growth parameters of tomato seedlings variety PKM-1 influenced by the seed imbibition on *M. aminovorans (*MtTm13), *M.thiocyanatum* (DSM 11490^{T}) under different PEG 6000 concentrations (0 to -3bars)

Treatments	Peg concentration (bars)		Speed of germination	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Vigor index
T_1 (Un-inoculated control)	0		1.91±0.009^{h}	17.2±0.14^{k}	1.7±0.009^{h}	1.1±0.01^{f}	2.2±0.24^{h}	48.3±0.03^{k}
	-0.5		0.86±0.007^{l}	7.9±0.02^{m}	1.3±0.014^{i}	0.6±0.007^{i}	1.9±0.02^{i}	14.9±0.22^{n}
	-1		1.21±0.02^{j}	10.5±0.02^{l}	1.4±0.02^{i}	0.6±0.01^{i}	2.0±0.02^{h}	21.2±0.06^{m}
	-1.5		0.6±0.002^{m}	4.0±0.02^{n}	0.7±0.02^{k}	1.0±0.002^{g}	1.8±0.02^{i}	7.2±0.04^{o}
T_2 (*M. aminovorans* -MtTm13)	0		2.3±0.01^{f}	24.0±0.02^{h}	2.7±0.007^{c}	1.1±0.01^{f}	3.8±0.02^{d}	91.2±0.50^{f}
	-0.5		2.1±0.02^{g}	24.0±0.43^{h}	1.2±0.02^{j}	0.6±0.007i	1.7±0.02^{j}	43.2±0.58^{l}
	-1		3.0±0.06^{c}	32.0±0.24^{d}	2.7±0.03^{c}	1.4±0.01^{c}	4.1±0.07^{c}	131.2±2.1^{b}
	-1.5		3.2±0.06^{b}	40.0±0.73^{b}	2.2±0.03^{f}	1.3±0.009^{d}	3.4±0.05^{e}	116.5±1.2^{d}
	-2		1.7±0.02^{i}	21.3±0.02^{i}	2.0±0.03^{g}	1.2±0.01^{e}	3.1±0.07^{f}	68.2±0.7^{i}
	-2.5		2.5±0.01^{e}	29.3±0.09^{e}	2.5±0.04^{d}	1.3±0.007^{d}	3.7±0.02^{d}	111.3±0^{e}
	-3		1.6±0.02^{i}	18.5±0.02^{j}	2.9±0.05^{b}	1.9±0.02^{b}	4.8±0.07^{b}	89.3±0.01^{f}
T_3 (*M. thiocyanatum* - DSM11490^{T})	0		4.1±0.01^{a}	42.7±0.09^{a}	2.7±0.03^{c}	1.3±0.02^{d}	4.0±0.02^{c}	170.8±1.6^{a}
	-0.5		3.0±0.04^{c}	33.3±0.19^{c}	2.4±0.04^{d}	1.0±0.007^{g}	3.3±0.02^{e}	113.2±1.3^{d}
	-1		2.3±0.03^{f}	26.7±0.19^{g}	2.3±0.02^{e}	0.9±0.01^{h}	3.1±0.07^{f}	85.4±0^{g}
	-1.5		2.8±0.03^{d}	28.0±0.53^{f}	1.6±0.02^{h}	1.3±0.01^{d}	2.9±0.02^{g}	81.2±0.92^{h}
	-2		2.7±0.009^{d}	24.0±0.48^{h}	4.1±0.07^{a}	1.0±0.002^{g}	5.1±0.02^{a}	122.4±2.3^{c}
	-2.5		2.1±0.02^{g}	21.2±0.09^{i}	1.7±0.002^{h}	1.0±0.01^{g}	2.6±0.02^{g}	57.5±0.73^{j}
	-3		1.1±0.005^{k}	10.6±0.02^{l}	1.2±0.01^{j}	2.7±0.005^{a}	3.8±0.07^{d}	41.7±0.77^{l}
Mean	T	T_1	0.65^{c}	5.6^{c}	0.73^{b}	0.46^{c}	1.11^{b}	13.0^{c}
		T_2	2.3^{b}	26.9^{a}	2.31^{a}	1.25^{b}	3.51^{a}	92.9^{b}
		T_3	2.5^{a}	26.6^{b}	2.28^{a}	1.30^{a}	3.54^{a}	96.0^{a}
	S	S_1	2.7^{a}	27.9^{a}	2.3^{a}	1.1^{c}	3.3^{a}	103.4^{a}
		S_2	1.9^{c}	21.7^{d}	1.6^{d}	0.7^{f}	2.3^{e}	57.0^{e}
		S_3	2.1^{b}	23.0^{c}	2.1^{b}	0.9^{d}	3.0^{b}	79.2^{b}
		S_4	2.1^{b}	23.9^{b}	1.5^{e}	1.1^{b}	2.6^{d}	68.2^{c}

		S_5	1.4^{e}	15.0^{f}	2.0^{c}	0.7^{f}	2.7^{c}	63.5^{d}
		S_6	1.5^{d}	16.8^{e}	1.4^{f}	0.8^{e}	2.1^{f}	56.2^{e}
		S_7	0.8^{f}	9.7^{g}	1.3^{f}	1.5^{a}	2.8^{c}	43.6^{f}
Grand Mean			1.85	19.75	1.77	1.00	2.72	67.3
SE d	T		0.02	0.2	0.02	0.008	0.04	0.62
	S		0.03	0.3	0.03	0.01	0.06	0.94
	T x S		0.05	0.5	0.06	0.02	0.11	1.64
CD (P=0.05)	T		0.04	0.4	0.04	0.02	0.08	1.25
	S		0.06	0.5	0.06	0.03	0.13	1.91
	T x S		0.19	1.0	0.11	0.05	0.23	3.31

Seeds were placed in sterile petridish, after 12 hours adding different PEG 6000 concentration (0 to -3 bars) and observe the growth parameters on 15th day. There is no growth observed on un-inoculated control (-2 to -3 bars). Each value represents mean ± SE of 3 replicates per treatment. In the similar column, significant differences at (P<0.05) levels are indicated by different letters. Data followed by similar letter in the similar column are not significantly different from each other. **T** = Treatments, **S** = PEG concentrations, un- inoculated control (**T1**), *Methylobacterium aminovorans* (MtTm13) (**T2**), *Methylobacterium thiocyanatum* (DSM11490T) (**T3**).

Table.2 Root hairs parameters of tomato seedlings variety PKM1 influenced by the seed imbibitions on *M. aminovorans* (MtTm13), *M.thiocyanatum* (DSM 11490^{T}) under different PEG 6000 concentrations

Particulars	(T_1) Control	(T_2) *M. aminovorans*	(T_3) *M.thiocyanatum*
Number of root hairs	430	450	441
Number of infected root hairs	-	255	223
Number of uninfected root hairs	430	195	218
Root hair length (µm)	104.4	80.2	72.4
Root hair breadth (µm)	2.4	2.9	3.2

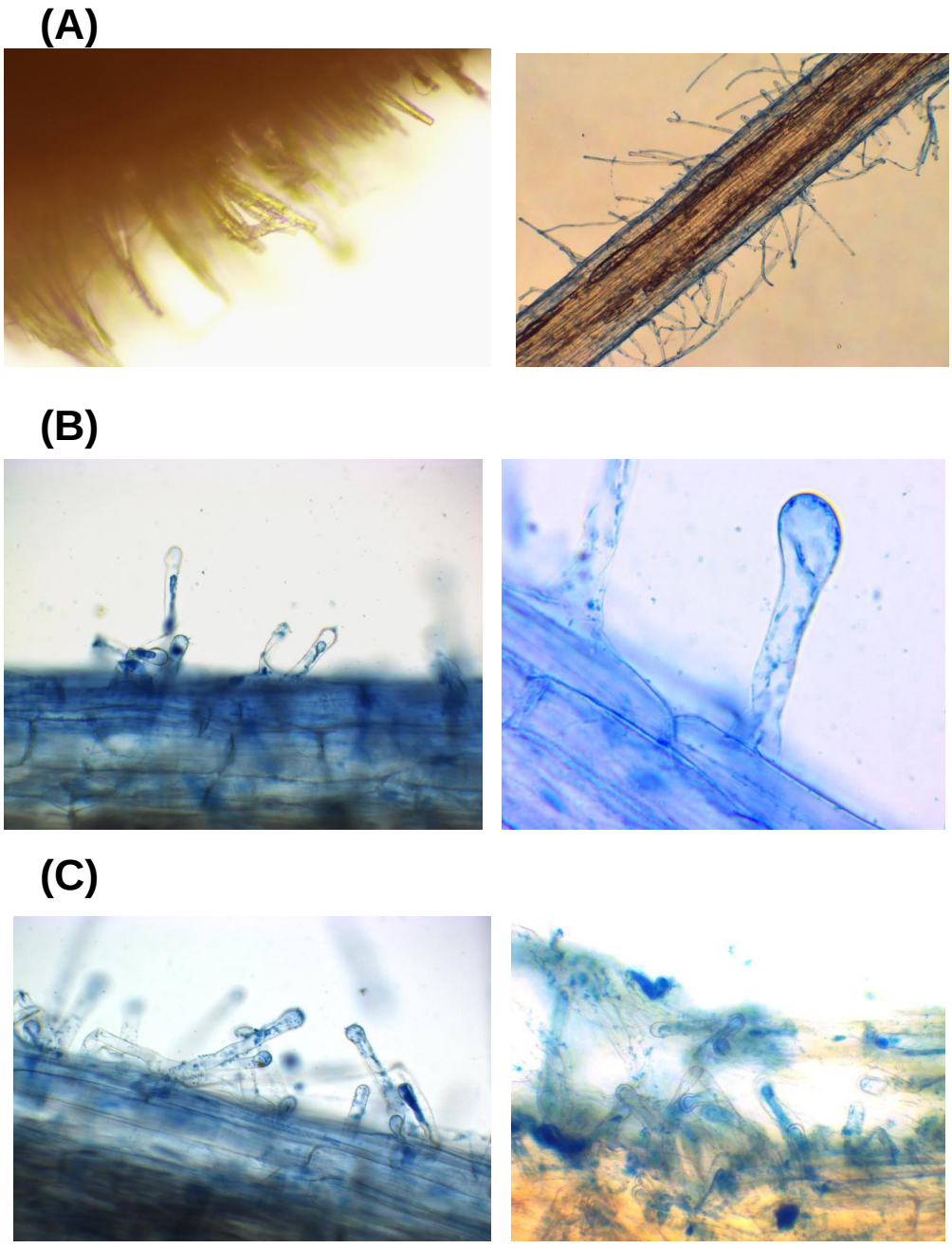
The surface sterilized tomato seeds were treated with *Methylobacterium* spp. and placed in moistened filter paper in sterile petridish and incubates at 25^{0}C. After 5-7days the lateral root of seedlings showed abundant root hair. All the root hairs were observed under microscope by mounting one root per presterilized slide where the entire root was observed to be not deformed. Thus, *Methylobacterium* spp. was inoculated to the seedlings after a period of day the roots stained by aniline blue/ lactophenol stain (White *et al.*, 2014) the deformed root hairs were observed. Root hair deformations were observed using bright field microscope. T = Treatments, un- inoculated control (T1), *Methylobacterium aminovorans* (MtTm13) (T2), *Methylobacterium thiocyanatum* (DSM11490T) (T3).

Table.3 Biochemical parameters of tomato seedlings variety PKM1 influenced by the seed imbibition on *M. aminovorans* MtTm13, *M.thiocyanatum* (DSM 11490^{T}) under different PEG 6000 concentrations

Treatments	PEG concentration (bar)	Proline (µmol g^{-1} fresh leaf tissue)	Glycine Betaine(µg g^{-1} fresh leaf tissue)	Catalase(µmolmin^{-1} g^{-1} of leaf tissues)	Peroxidase(min^{-1} g^{-1} of leaf tissues)	SOD(µg^{-1} protein min^{-1} of leaf tissue)
T_1 (Un-inoculated control)	-	3.42 ±0.026^{c}	0.19±0.007^{c}	1.03±0.002^{a}	1.24±0.01^{a}	16.2±0.27^{a}
T_2 (*M. aminovorans* - MtTm13)	-1.5	3.96±0.079^{a}	0.62±0.002^{b}	0.28±0.005^{c}	0.11±0.00^{c}	11.5±0.17^{c}
T_3 (*M. thiocyanatum* - DSM11490^{T})	-2	3.77 ±0.008^{b}	0.75±0.006^{a}	0.35±0.006^{b}	0.16±0.00^{b}	12.8±0.18^{b}
Grand mean		3.71	0.52	0.55	0.50	13.5
SEd		0.084	0.01	0.008	0.01	0.37
CD (P=0.05)		0.20	0.04	0.020	0.02	0.91

Seeds were placed in sterile petridish, after 12 hours adding PEG 6000 concentration of (-1.5 bar in T_2 & -2 bar in T_3)and T_1 as un-inoculated Control without adding PEG 6000 concentration and then observations were taken on 15 th day. Each value represents mean ± SE of 5 replicates per treatment. In the similar column, significant differences at (P<0.05) levels are indicated by different letters. Data followed by similar letter in the similar column are not significantly different from each other. T = Treatments, S = PEG concentrations, un- inoculated control (T1), *Methylobacterium aminovorans* MtTm13 (T2), *Methylobacterium thiocyanatum* (DSM11490T) (T3).

Fig.1 Tomato root hair infection and deformation assay observed under Bright field microscope @ 40X magnification (a) Un-inoculated control roots shown long and slender root hairs under induced moisture stress tolerance. Tomato root hairs infected by (b) *Methylobacterium aminovorans* (c) *Methylobacterium thiocyanatum* under induced moisture stress toleranceshowing root tip bulging and thick short root hair



Inoculated *M.thiocyanatum* (3.2µm) exhibit higher root hair breadth followed by *M. aminovorans* (2.9µm) than un-inoculated control (2.4µm) presented in table 2.

Effect of PEG -6000 (0 to -3bars) induced osmotic stress on antioxidant enzymes

The results pertaining to the effect of PEG-6000 induced water stress on superoxide dismutase (SOD), catalase (CAT) and peroxidase (PER) are summarized in table 3. Antioxidant enzymes play a major role in reducing the content of reactive oxygen species in plants which produced under stress condition. Earlier, several studies have shown the role of antioxidant enzymes for stimulating plant growth under salt stress condition (Subramanian *et al.,* 2016). The activities of antioxidant enzymes examined in this study (SOD, POD, and CAT) increased significantly in the inoculated tomato seedlings under moisture stress compared to the un-inoculated control. The minimum growth of the seedlings was recorded up to PEG concentration (0 to -1.5 bars) and no growth was recorded in remaining concentration (-2 to -3 bars) in un-inoculated control which shows that the seedlings were exposed to severe drought osmotic stress by inducing PEG.

Then the *Methylobacterium spp.* inoculated treatments showed the growth of the seedlings in all PEG concentration (0 to -3 bars) whereas based on the maximum speed of germination and germination percentage the more vigorous growth was observed in -1.5 bars of *M. aminovorans* and -2 bars of *M. thiocyanatum* based on seedling length. It shows that the seedlings has the capability to withstand osmotic stress, these two different concentrations were further processed to analyze antioxidant enzyme activity. The catalase activity did not significantly differ among the treatments under a non-stressed condition which showed the decreased activity of catalase enzyme. The CAT activity increased under the drought condition was found in inoculated *M. aminovorans* (0.28µmolmin^{-1} g^{-1} of leaf tissues) followed by *M. thiocyanatum* (0.35µmolmin^{-1} g^{-1} of leaf tissues) given in table 3. The least catalase activity was found in control (1.03µmolmin^{-1} g^{-1} of leaf tissues). The results are in accordance with finding of Kumar *et al.,* (2017) reported that *Bacillus altitudinis* and *Methylobacterium* spp. treated rice plants showed more catalase activity than control under drought stress. Impact of PPFM enhanced the catalase activity on tomato under drought condition (Chandrasekaran *et al.,* 2017). The catalase activity increased in crops resulted from the foliar application of PPFM (Sivakumar *et al.,* 2017). The treated *M. aminovorans* (0.11min^{-1}g^{-1} leaf tissue) and *M. thiocyanatum* (0.16 min^{-1}g^{-1} leaf tissue) peroxidase values were reduced as compared to un-inoculated control (1.24 min^{-1}g^{-1} leaf tissue) given in table 3.The results were in accordance with the finding of Madhaiyan *et al.,* (2006) showed that the inoculation of *Methylobacterium* sp., significantly enhanced the peroxidase enzymes on groundnut. POD content in the rape leaves treated with PGPR strains were decreased as compared with control (Ren *et al.,* 2019). Catalase and peroxidase plays a significant role in the cell controlling by activating and deactivating of reactive oxygen species and also it was possible to generate many apoplastic enzymes under normal & stress conditions (Sairam *et al.,* 2005).The unscavanged H_2 O_2 diffused from sorghum chloroplast to cytosol, efficiently scavenged by POX and CAT (Zhang and Kirkham 1996). SODs are a group of metalloenzymes that are considered to be the first defense against ROS that dismute ROS into H_2O_2 formed during stress. The level of SOD activity decreased under PEG concentration of inoculated *M. aminovorans* (11.5µg^{-1} protein min^{-1}leaf tissue) and *M. thiocyanatum* (12.8µg^{-1} protein min^{-1}leaf

tissue) as compared to un-inoculated control (16.2µg^{-1}leaf tissue protein min^{-1}) given in table 3.The results were in accordance with the treated *Methylobacterium* strain in snap bean were enhancing the antioxidant enzyme activity of SOD with PPFM significantly reduced when compared with control (Abd El-Gawad *et al.,* 2015). The enhanced activity of antioxidant enzymes are CAT, PER, SOD on tomato seedlings due to beneficial impacts of Methylobacterial treatments towards improved plant performance under moisture stress condition.

Effect of PEG -6000 (0 to -3bars) induced osmotic stress on osmolytes

The plant accumulates many compatible osmolytes under stress conditions, such as proline, glycine betaine (GB), starch, alcohol and soluble protein (Delauney and Varma 1993). The degree of stress tolerance in a variety of crop plants and enzymes involved in stress tolerance has been positively associated with levels of organic solutes like proline and GB (Garg and Noor 2009). The proline content was significantly influenced by both drought stress and Methylobacterial treatments. A considerable increased in the amount of free proline was observed in all the treatments due to drought stress. However, it was interesting note that *M. aminovorans* treated seedlings produced the highest concentration of proline (3.96µmol g^{-1} fresh leaf tissues) relative to *M. thiocyanatum* (3.77µmol g^{-1} fresh leaf tissues) and control (3.42µmol g^{-1} fresh leaf tissues) presented in table 3. Our results were supported by Sivakumar *et al.,* (2017) showed PPFM treated tomato plants exhibit higher proline content when compared to un-inoculated control under drought conditions. In addition to this Ruiz-Sanchez *et al.,* (2011) reported the Arbuscular mycorhiza and *Azospirillum* inoculation improved the content of proline in rice under drought conditions as compared to control. The inoculated *Bacillus* sp., on plant showed higher proline content and it had a greater tolerance to water stress conditions (Gusain *et al.,* 2015).The drought stress induced by PEG-6000 enhancing the endogenous proline concentrations in tomato (Shtereva *et al.,* 2008). The increased proline content, especially in tolerant genotypes for osmotic stress imposition by PEG, may be attributed to the multifunctional role of proline as a signaling molecule for modulating mitochondrial functions, controlling cell proliferation and triggering different gene expression, which may be necessary for plants to recovery from stress (Szabados and Savoure 2009).The treated *Methylobacterium* seedlings exhibit maximum enzyme activity than un-inoculated control. As a result the higher GB content were observed in *M.thiocyanatum* (0.75 µg g^{-1} fresh leaf tissue) followed by *M.aminovorans* (0.62µg g^{-1} fresh leaf tissue) while compared to un-inoculated control (0.19µg g^{-1} fresh leaf tissue) presented in table 3. Similarly, inoculated PGPR strains in maize plant accumulated significantly higher GB content than un-inoculated control under drought stress conditions (Sandhya *et al.,* 2010). Therefore thus, the *Methylobacterium* spp. might be used in reducing the negative impact of drought stress and enhancing the growth production of tomato seedlings.

The present study showed that *Methylobacterium* spp. was capable of mitigating the moisture stress induced by PEG and also enhancing the seed germination, vigor index and antioxidant enzymes. It seems that *Methylobacterium* spp. influenced the biochemical parameters of tomato seedlings and helped them improving drought tolerance. *Methylobacterium* spp. proved to have a promising role in improving plant performance under drought condition. In conclusion, in recent year, the agricultural production was drastically reduced due to drought stress. For this dispute, use of *M.*

aminovorans (MtTm13) and *M. cyanatum* (DSM11490T) mitigate the early growth stages of drought in tomato seedlings and its enhance the plant growth promoting parameters by inducing antioxidant enzymes under induced moisture stress condition.

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