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Characterization of phyllosphere methylotrophic bacteria isolated from the groundnut and their impact on growth, yield and quality of the kernel

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Abstract

Aim : The present study was undertaken to characterize and evaluate the habitat adopted methylotrophic bacteria on groundnut growth and yield at field level.

Methodology : Nine elite methylotrophic bacterial isolates were selected based on early plant growth promotion and quantified for plant growth promoting (PGP) traits along with one standard strain, *Methylobacterium thiocyanatum* DSM 11490^T. Two field experiments were carried out with eleven different treatments arranged in a randomized block design with three replications. Bacterial inoculant was applied as seed treatment as well as foliar application at 35 and 70 days after sowing. Plant growth and yield parameters were estimated periodically and after harvest.

Results : All the tested isolates exhibited multiple PGP traits. In Vriddhachalam, the yield of groundnut was significantly higher in *Methylobacterium thiocyanatum* VRI7-A4 (1688.9 kg ha⁻¹) followed by *Paenibacillus hunanensis* COG-4 (1675.8 kg ha⁻¹) compared to un inoculated control treatment. In Tindivanam, yield content of groundnut was found significantly higher in *Methylobacterium thiocyanatum* VRI7-A4 (1678.6 kg ha⁻¹) followed by *Pseudomonas psychrotolerans* K-TMV7-6 (1667.5 kg ha⁻¹). In addition, *Methylobacterium* inoculation increased the kernel protein content as compared to the control treatment in both the field experiments.

Interpretation : The results showed that *Methylobacterium populi* TMV7-4, *Methylobacterium thiocyanatum* VRI7-A4, *Pseudomonas psychrotolerans* K-TMV7-6 may be used as bio-inoculant for groundnut crop.

Key words: Groundnut, Kernel quality, Methylotrophic bacteria, *mxhF* gene

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Introduction

Legumes are considered as the third largest family of higher plants, where groundnut (*Arachis hypogaea* L.) is one of the most vital crops of this family. Groundnut is considered as one of the important crop both economically as well as agriculturally (Krishna et al., 2015). India is one of the leading countries in groundnut production next to China. However, a declining trend in productivity has been observed since 2013 (Economic survey, 2016–2017). In India, area coverage, production and yield of groundnut during 2012–2013 reduced by 0.49 million ha, 2.21 million tons and 415 kg ha⁻¹, respectively against corresponding period of 2011–2012. In Asian countries, bio-inoculants are included as one of the major component in the integrated soil fertility management programs. Increased use of bio-inoculants results in environmental friendly soil fertility management with the potential of sustainable agriculture development compared to the continuous application of chemical fertilizers alone (Sharma et al., 2013). Use of bio-inoculants for plant nutrition in addition to chemical fertilizer is gaining more importance at field level. Researchers are using microbial inoculants successfully at field level as they have been potentially recognized for stimulating and increasing plant growth and yield (Mukhongo et al., 2017; Bradacova et al., 2019).

Several studies have demonstrated that the combined use of microbial inoculants and reduced / recommend chemical fertilizers can sustain soil fertility and crop yield (Bhardwaj et al., 2014). With the aim of improving plant growth many different types of bio-inoculants have been developed, but most of them are not crop specific. Due to non-crop specific nature of bio-inoculants, microorganisms are failed to colonize the plant effectively and their full potential are not achievable (Itelima et al., 2018). To obtain maximum benefits out of bio-inoculants, more attention has to be paid for the selection of efficient crop specific bio-inoculants (Sharma et al., 2009). Habitat adopted methylo-trophic bacterial isolates obtained from the phyllosphere of groundnut plants grown in different geographical location of Tamil Nadu were initially tested for early growth promotion in groundnut. Based on the results obtained from the previous study (Krishnamoorthy et al., 2018,) efficient isolates were selected and tested under field level. Keeping in view the above, the objective of this study was to evaluate and select efficient methylo-trophic bacterial isolates for enhancing groundnut growth, yield and quality parameters for formulating a groundnut specific bio-inoculant.

Materials and Methods

Test Microorganisms: Nine methylo-trophic bacterial isolates were selected from the isolates obtained from phyllosphere of groundnut leaf samples obtained from different geographical locations of Tamil Nadu in addition one standard Methylo-trophic bacterial strain, *Methylobacterium thiocyanatum* DSM 11490^T (kind gift from Prof. Tongmin Sa, Chungbuk National University, South Korea) was also used in this study.

Estimation of plant growth promoting traits: Indole acetic acid production of methylo-trophic isolates was quantitatively measured by the method described by Bric et al. (1991). Nitrogen fixing efficiency of the isolates was evaluated by using acetylene reduction assay (ARA). The ARA was calculated and expressed as p moles of ethylene formed per mg of protein (David et al., 1980). ACC deaminase activity was determined by growing the bacterial isolates on nitrogen-free medium amended with 3 mM ACC as nitrogen source (Penrose and Glick, 2003) and the amount of α -ketobutyrate produced by the enzymatic hydrolysis of ACC was estimated. The phosphate solubilization of methylo-trophic isolates were quantitatively determined by inoculation in Pikovskaya's broth supplemented with 0.5 % tricalcium phosphate. The phosphorous content was analyzed by adopting the method of Bray and Kurtz (1945). Exopolysaccharides (EPS) was extracted and quantified by measuring the dry weight (Hebbar et al., 1992) and glucose was measured by phenol-sulphuric acid method (Dubois et al., 1956).

Methanol oxidizing (mx) gene amplification: The presence of *mx*A gene in the bacterial isolates were authenticated by using the gene specific primers. The forward primer *mx*A-F-1003 (5'-GCGGCACCAACTGGGGCTGGT-3') and reverse *mx*A-R-1561 (5'-GGGCAGCATGAAGGGCTCCC-3') of 550 bp were used (McDonald and Murrell, 1997). The amplification was carried out in 100 μ l aliquots by mixing 50–90 ng template DNA with the PCR buffer (10 x); 100 μ M (each) dATP, dCTP, dTTP and dGTP; primers (100 ng each) and 1.0 U Taq polymerase. PCR amplification was performed in a thermocycler using following conditions: initial denaturation at 94°C for 5 min, 35 cycles consisting of 95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension 72°C for 5 min (Meena et al., 2012). PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide (10 μ l) and documented. The results showed the presences of *mx*A gene in all the tested methylo-trophic bacterial isolates.

Formulation of liquid bio-inoculant: Microbial formulations were prepared with all the ten methylo-trophic bacteria. The cultures were grown in the glycerol peptone liquid medium for 48 hrs (~1 x 10⁹ CFU ml⁻¹). Liquid bio-inoculant was developed using these bacterial cultures with 0.1% CMC as sticker and 1% glycerol as a preservative.

Treatment details and plant biometric parameters: Field level evaluation of ten methylo-trophic bacteria were performed in two different locations in Tamil Nadu. One is in Oil Seed Research Station, Tamil Nadu Agricultural University (TNAU) Tindivanam and another one is in Regional Research Station, Vriddhachalam, TNAU, Tamil Nadu, India. All the ten methylo-trophic bacterial liquid bio-inoculant formulation were used for seed treatment. Totally 11 treatments (T1 – *Acinetobacter baumannii* K-TMV7-7; T2 – *Pantoea stewartii* subsp. indologenes N-CO6-5; T3 – *Pantoea dispersa* ICGV-2; T4 – *Methylobacterium populi* TMV7-4; T5 – *Methylobacterium thiocyanatum* VRI7-A4; T6 – *Methylobacterium radiotolerans* VRI8-A4; T7 – *Pseudomonas*

psychrotolerans K -TMV7-6; T8 – *Paenibacillus hunanensis* COG-4; T9 – *Rhizobium pusense* TMV2-6; T10 – *Methylobacterium thiocyanatum* DSM11490T; T11–Control) with three replications arranged in a randomized block design.

In Vriddhachalam, the plot size was 3 x 4 M, while in Tindivanam the plot size was 4 x 5 M. In Oil Seed Research Station, Tindivanam Western 44 variety and in Regional Research Station, Vriddhachalam VRI 8 varieties were used. Methylophilic bacteria were used in seed treatment (1%) during sowing and foliar application (@ 10 ml l⁻¹) at 35 and 70 days after sowing. Continuous monitoring was made and followed blanket recommendation of fertilizers (25, 50, 75 and 60 kg ha⁻¹ of N, P, K and Sulphur, respectively). Plants were harvested at their physiological maturity. Growth and yield parameters were tested after harvesting. The crude protein content was evaluated by digestion of the sample using Kjeldahl's method (Devani et al., 1989) and oil content of the kernel was estimated by Soxhlet extraction method (Maranz et al., 2003).

Statistical analyses: The data were subjected to analysis of variance (ANOVA), and the significant of differences between the means was determined by Duncan's Multiple Range Test (DMRT) at $p < 0.05$. All of the data were analyzed using IBM SPSS Statistics 20.

Results and Discussion

Many of the plant beneficial bacteria are plant species specific and establish close association with the similar plant species. In a previous study, crop specific methylophilic bacteria were isolated and screened for groundnut early plant growth promotion (Krishnamoorthy et al., 2018). In continuation with previous study (Krishnamoorthy et al., 2018), , nine efficient isolates were used at field level (in two locations) to develop a crop specific bio-inoculant along with one standard methylophilic strain. All the tested ten isolates produced various

amount of IAA and EPS. Higher IAA and EPS were produced by *Pantoea stewartii* subsp. indologenes N-CO6-5 (6.6 µg ml⁻¹) and *Pantoea dispersa* ICGV-2 (4.5 µg ml⁻¹), respectively (Table 1).

IAA production by the phyllosphere bacteria were previously reported (Akter et al., 2016). Higher ARA activity was found in the *Pseudomonas psychrotolerans* K-TMV7-6 (2546 pmol of ethylene/mg protein/h). *Rhizobium pusense* TMV 2-6 showed higher ACC deaminase activity and higher amount of P released by *Pantoea dispersa* ICGV-2 and *Paenibacillus hunanensis* COG-4 (2.62 g/l) from insoluble P source. Similarly, P solubilization nature of *Pantoea* and *Paenibacillus* was reported (Hernandez et al., 2018). Nitrogen is one of the major nutrients required by the plants for their proper cell functions. Nitrogen fixing ability of methylophilic isolates were previously reported from *Jatropha curcas* (Madhaiyan et al., 2015). In this study, 56 percent of the isolates exhibited nitrogen fixing ability. ACC deaminase-containing methylophilic bacteria could reduce a significant portion of the plant damage by breaking down the ethylene precursor in to ammonia and α-ketobutyrate. Bacteria which possess ACC deaminase was well proven to mitigate stress in various plants (Win et al., 2018; Saikia et al., 2018).

In this study, 78 percent of the isolates were found to produce ACC deaminase which might help in mitigating stress in plants. Phosphorus is one of the major essential macronutrients required for plant growth and development, but in soil most of the phosphorus are converted to insoluble form by reacting with other soil ions. Jayashree et al. (2011) reported the production of organic acids and enzymes for solubilization of insoluble phosphorus by methylophilic isolates. Qualitative plant growth promoting traits of total 80 methylophilic bacterial isolates data were given in the previous report (Krishnamoorthy et al., 2018). However, it is very hard to select efficient isolate with multiple plant growth promoting traits only with the qualitative data. Hence, quantitative estimation of plant growth promoting traits were performed in this study. In addition, to confirm the methanol

Table 1: Quantitative estimation of multiple PGP traits of selected methylophilic bacterial isolates

Isolate	IAA production (µg ml ⁻¹)	ARA activity (pmol mg ⁻¹ protein hr ⁻¹)	ACC deaminase (nmolµg/ protein hr ⁻¹)	P solubilization (g l ⁻¹)	EPS production (µg ml ⁻¹)
<i>Acinetobacter baumannii</i> K-TMV7-7	3.1 ± 0.4	ND	ND	2.59 ± 0.36	3.3 ± 0.25
<i>Pantoea stewartii</i> subsp. indologenes N-CO6-5	6.6 ± 0.9	ND	0.13 ± 0.005	2.41 ± 0.33	3.5 ± 0.04
<i>Pantoea dispersa</i> ICGV-2	5.1 ± 0.7	508 ± 175	0.90 ± 0.06	2.62 ± 0.36	4.5 ± 0.36
<i>Methylobacterium populi</i> TMV7-4	4.1 ± 0.6	ND	2.40 ± 0.09	ND	3.4 ± 0.16
<i>Methylobacterium thiocyanatum</i> VRI7-A4	3.3 ± 0.4	58 ± 1.9	0.22 ± 0.007	ND	4.1 ± 0.04
<i>Methylobacterium radiotolerans</i> VRI8-A4	2.5 ± 0.3	ND	ND	ND	3.1 ± 0.01
<i>Pseudomonas psychrotolerans</i> K-TMV7-6	4.9 ± 0.7	2546 ± 436	1.39 ± 0.04	2.44 ± 0.33	4.2 ± 0.12
<i>Paenibacillus hunanensis</i> COG-4	4.1 ± 0.6	193 ± 58	0.73 ± 0.08	2.62 ± 0.36	3.1 ± 0.01
<i>Rhizobium pusense</i> TMV 2-6	3.6 ± 0.5	271 ± 86	3.09 ± 0.08	0.32 ± 0.04	4.8 ± 0.04
<i>Methylobacterium thiocyanatum</i> DSM11490T	3.3 ± 0.5	1816 ± 314	0.26 ± 0.04	2.59 ± 0.35	3.1 ± 0.01

Data are mean of five replicates ± S.E.; IAA - Indole acetic acid; ARA - acetylene reduction assay; ACC deaminase-1-aminocyclopropane-1-carboxylate deaminase; EPS - Exopolysaccharides; ND - not detected

Table 2: Effect of methyilotrophic bacterial isolates on groundnut yield and kernel quality under field condition after 75 days of sowing

Treatments	Vriddhachalam			Tindivanam		
	Pod yield (kg ha ⁻¹)	Oil percentage	Protein (%)	Pod yield (kg ha ⁻¹)	Oil percentage	Protein (%)
T1 - <i>Acinetobacter baumannii</i> K-TMV7-7	1567.5±14.6 ^a	49.8±1.2 ^a	24.6±1.0 ^c	1591.7±15.8 ^a	47.6±0.6 ^a	22.6±1.2 ^a
T2 - <i>Pantoea stewartii</i> subsp. <i>indologenes</i> N-CO6-5	1580.0±16.3 ^a	49.0±0.6 ^a	22.4±0.8 ^a	1585.0±10.1 ^a	46.5±0.2 ^a	23.9±0.6 ^a
T3 - <i>Pantoea dispersa</i> ICGV-2	1563.3±13.1 ^a	49.2±1.1 ^a	26.6±1.8 ^c	1589.2±8.8 ^a	47.2±1.2 ^a	25.6±1.1 ^b
T4 - <i>Methylobacterium populi</i> TMV7-4	1632.6±32.9 ^b	50.1±0.6 ^a	23.5±0.9 ^{ab}	1643.4±35.5 ^b	48.5±2.1 ^a	25.0±0.6 ^b
T5 - <i>Methylobacterium thiocyanatum</i> VR17-A4	1688.9±16.9 ^b	51.0±1.6 ^a	26.0±1.2 ^c	1678.6±41.4 ^b	48.0±1.0 ^a	26.8±1.8 ^b
T6 - <i>Methylobacterium radiotolerans</i> VR18-A4	1581.7±22.4 ^a	49.9±0.5 ^a	24.0±0.4 ^b	1605.0±8.8 ^{ab}	47.9±0.6 ^a	25.6±0.9 ^b
T7 - <i>Pseudomonas psychrotolerans</i> K-TMV7-6	1647.2±26.5 ^b	50.0±1.8 ^a	24.2±0.3 ^b	1667.5±33.6 ^b	49.3±0.5 ^a	24.2±0.3 ^b
T8 - <i>Paenibacillus hunanensis</i> COG-4	1675.8±19.3 ^b	49.8±0.7 ^a	20.1±1.1 ^a	1599.2±2.2 ^a	48.0±0.2 ^a	22.1±1.9 ^a
T9 - <i>Rhizobium pusense</i> TMV 2-6	1529.7±21.9 ^a	49.6±0.6 ^a	24.4±0.4 ^b	1588.3±6.7 ^a	47.2±0.6 ^a	20.4±0.6 ^a
T10 - <i>Methylobacterium thiocyanatum</i> DSM11490T	1574.5±12.3 ^a	49.5±1.8 ^a	24.8±1.0 ^b	1592.5±11.8 ^a	47.0±0.8 ^a	25.5±1.5 ^b
T11 - Control	1562.3±6.9 ^a	49.5±1.6 ^a	24.0±0.8 ^b	1592.5±5.2 ^a	47.3±0.9 ^a	22.9±0.9 ^a

Data are mean of five replicates±S.E; p < 0.05 according to DMRT, different letters indicate difference among treatments

utilizing ability of the isolates, presence of methanol dehydrogenase (MDH) gene in the isolates was identified by gene amplification study. The enzyme MDH involved in oxidizing methanol to formaldehyde, the intermediate during the metabolism in methyilotrophs. The gene encoding α subunit of MDH is *mxoF*, which is highly conserved functional gene often used for methyilotrophic diversity analysis (Lau *et al.*, 2013). The presence of *mxoF* gene (~550bp) in all the ten methyilotrophic isolates used in this study is presented in Fig. 1. Similarly the presence of *mxoF* gene was detected in methyilotrophic isolate obtained from wheat (Meena *et al.*, 2012), *Anadenanthera colubrina* (Alibrandi *et al.*, 2018) and mustard plant (Subhaswaraj *et al.*, 2017).

In Vriddhachalam, significant increase in shoot length was observed in T4 – *Methylobacterium populi* TMV7-4, T5 – *Methylobacterium thiocyanatum* VR17-A4, T6 – *Methylobacterium radiotolerans* VR18-A4 and T7 – *Pseudomonas psychrotolerans* K-TMV7-6 and the nodule number was significantly higher in the plants treated with T5 – *Methylobacterium thiocyanatum* VR17-A4 and T7 – *Pseudomonas psychrotolerans* K-TMV7-6 (Fig. 2A). Significantly higher root length and dry weight was observed in T4 – *Methylobacterium populi* TMV7-4 and T7 – *Pseudomonas psychrotolerans* K-TMV7-6 (Fig. 2B). Plant root biomass might be directly correlated with the amount of nutrition and water uptake from soil. Recently Klikno and Kutschera (2017) illustrated that phytohormones produced by *Methylobacterium* significantly increases the root biomass. Similarly, in this study the methyilotrophic bacteria used was found to produce phytohormone auxin and all the isolates enhanced root biomass at field level. In connection with this role of IAA produced by *Methylobacterium extorquens* MP1 and *Methylobacterium zatmanii* MS4 in plant root development was well documented (Pattnaik *et al.*, 2017). Even though the plant colonization was not accessed in this study, the increased plant parameters observed

in all inoculation treatments indicated that methyilotrophic bacteria had effectively established in association with the groundnut plants, in order to express its growth-promotion activities.

Fig.1: *mxoF* gene amplification in methyilotrophic bacterial isolates. L - 1000bp ladder; N-Negative control; 1-*Acinetobacter baumannii* K-TMV7-7; 2-*Pantoea stewartii* subsp. *indologenes* N-CO6-5; 3- *Pantoea dispersa* ICGV-2; 4-*Methylobacterium populi* TMV7-4; 5-*Methylobacterium thiocyanatum* VR17-A4; 6-*Methylobacterium radiotolerans* VR18-A4; 7-*Pseudomonas psychrotolerans* K-TMV7-6; 8-*Paenibacillus hunanensis*COG-4; 9 - *Rhizobium pusense* TMV2-6; 10 -*Methylobacterium thiocyanatum* DSM11490T.

Fig. 2: Effect of methylotrophic bacterial isolates on groundnut growth under field condition after 75 days of sowing at Vriddhachalam (A, C) and Tindivanam (B, D). 1–*Acinetobacter baumannii* K-TMV7-7; 2–*Pantoea stewartii* subsp. indologenes N-CO6-5; 3–*Pantoea dispersa* ICGV-2; 4–*Methylobacterium populi* TMV7-4; 5–*Methylobacterium thiocyanatum* VR17-A4; 6–*Methylobacterium radiotolerans* VR18-A4; 7–*Pseudomonas psychrotolerans* KTMV7-6; 8–*Paenibacillus hunanensis* COG-4; 9–*Rhizobium pusense* TMV2-6; 10–*Methylobacterium thiocyanatum* DSM11490T. shoot length and nodule number (A, B); Root length and dry weight (C, D) Data are mean of five replicates $\pm$ S.E; $p < 0.05$ according to DMRT, different letters indicate difference among treatments.

In Tindivanam, shoot length and nodule numbers were found to be higher in T2 – *Pantoea stewartii* subsp. indologenes N-CO6-5, T4–*Methylobacterium populi* TMV7-4, T5 – *Methylobacterium thiocyanatum* VR17-A4 and T7 – *Pseudomonas psychrotolerans* K-TMV7-6 at 75 DAS (Fig. 2C). Similarly, the dry weight was found to significantly improved by T2 – *Pantoea stewartii* subsp. indologenes N-CO6-5, T5–*Methylobacterium thiocyanatum* VR17-A4 and T7 – *Pseudomonas psychrotolerans* K-TMV7-6 treatments (Fig. 2D). Also *Methylobacterium* inoculation significantly increased the biomass accumulation in *Jatropha curcas* (Madhaiyan et al., 2015).

In Vriddhachalam, the yield of the groundnut was found significantly higher in *Methylobacterium thiocyanatum* VR17-A4 followed by *Paenibacillus hunanensis* COG-4 and *Pseudomonas psychrotolerans* K-TMV7-6 compared to uninoculated control treatment (Table 2). Previous reports demonstrated the methylotrophic bacteria mediated yield increases (Madhaiyan et al., 2015; Ardanov et al., 2016; de Aquino et al., 2018). A field experiment conducted by Ardanov et al. (2016) showed up to 20 percent yield increase in potato due to the inoculation of *Methylobacterium* sp. There were no significant variations in kernel oil content among the treatments, however, these parameters were found to be higher in *Methylobacterium thiocyanatum* VR17-A4 (51%). In line with this, Liu et al. (2017) reported that bacterial inoculation not influenced on groundnut kernel oil and protein content at field level experiment. However,

inoculation of *Rhizobium* was found to increase the groundnut oil content compared to the control treatment (Anandham et al., 2007; Hasan and Sahid, 2016). These variations might be caused by many factors such as plant genotype, other biotic and abiotic factors. Crude protein content was found higher in *Pantoea dispersa* ICGV-2 followed by *Methylobacterium thiocyanatum* VR17-A4 followed by *Methylobacterium thiocyanatum* DSM11490T. Similarly increase in protein content of groundnut kernel was observed in bacteria inoculated treatment compared to uninoculated treatment (Hasan and Sahid, 2016). In Tindivanam, the yield content of the groundnut was found significantly higher in *Methylobacterium thiocyanatum* VR17-A4 followed by *Pseudomonas psychrotolerans* K-TMV7-6 and *Methylobacterium populi* TMV7-4 compared to uninoculated control treatment. Similarly, *Methylobacterium* inoculation was found to increase the yield of rice and barely at field level compared to the control treatment (Tani et al., 2015). Yield increased by inoculation of methylotrophic bacteria might be due to the cumulative effect of multiple plant growth promoting traits exhibited by these isolates. There were no significant variations in kernel oil content noted among the treatments in Tindivanam, however, the crude protein content was found higher in *Methylobacterium thiocyanatum* VR17-A4 followed by *Pantoea dispersa* ICGV-2 and *Methylobacterium radiotolerans* VR18-A4.

The results of this field studies showed that *Methylobacterium populi* TMV7-4, *Methylobacterium*

thiocyanatum VRI7-A4, *Pseudomonas psychrotolerans* K-TMV7-6 inoculants increased groundnut growth and yield compared to control treatment. Hence, these three isolates may be recommended as crop specific bio-inoculant for groundnut after multi-location and on farm trials.

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