Research Article

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Isolation and *in vitro* screening of Pink Pigmented Facultative *Methylobacterium* (PPFMs) isolates for the production of phytohormones and ACC deaminase activity from the tea plantations of South India

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Abstract

Pink Pigmented Facultative *Methylobacterium* (PPFMs) strains were considered as highly efficient growth-promoting bacteria and are ACC deaminase producers which mitigate the drought impact in crop plants. Hence, PPFM strains were isolated from phyllosphere of the tea plantations of south India. A total of 253 bacterial isolates were obtained by using Methanol-AMS medium adopting standard methods. Among the 253 isolates, 105 potential isolates were screened for the production of plant growth hormones *viz.,* IAA, GA_3, carotenoids, and 35 potential isolates for ACC deaminase activity. Among 35 isolates tested, 5 isolates *viz*., MBVPR19D.L23 (51.7 µg/mL), MBANML10H.L06 (45.1 µg/mL), MBVPR19D.L22 (42.0 µg/mL), MBANML10H.L11 (40.5 µg/mL) and MBVPR UPASI L.An H.L13 (40.0 µg/mL) were found to produce higher level of IAA, GA_3, and Carotenoids. The highest activity of ACC deaminase was registered in the isolates of Central Travancore namely MBVPR19D.L22 and MBVPR19D.L23 along with one isolate MBKDMVG26H.L37 from Karnataka. The selected strains of MBVPR19 D.L22/23 were identified as *Methylobacterium radiotolerans* (OL440712) using 16s rRNA molecular technique. This is the first report of isolation of genus *Methylobacterium* from the acidic environment of tea ecosystem in South India. The present study also suggest that plant growth hormones and ACC deaminase enzyme activity of *Methylobacterium* species could play a critical role in mitigating the drought stress for a sustainable productivity in tea.

Keywords: Tea-*Camellia sinensis* , PPFM isolation , Phyllosphere, *in vitro* screening, Phytohormones, ACC deaminase

Introduction

In recent years, biotic and abiotic stresses especially pests, diseases, and drought are the major concerns in the tea plantations. In south India, 20% of the tea growing areas are severely affected by drought (Manivel *et al.,* 1994) which severely affects both the productivity and the quality of the tea (Zheng *et al.,* 2016). Abiotic stress influences the biochemical compositions of the tea plants including phytohormones such as Indole acetic acid (IAA), Cytokinins (Cks), Gibberellin (GA_3) (Bano *et al.,* 1994, Rivero *et al*., 2007 and Farooq *et al.,* 2009). Subsequently, higher production of ethylene resulting in senescence (Ma *et al*., 2003 and Arshad *et al*., 2008) can be inhibited by methylobacterial enzyme, ACC-deaminase that converts 1-aminocyclopropane-1-carboxylate (ACC) into α-ketobutyrate and ammonia. Hence, the concentration of ethylene is lowered indirectly enhancing the synthesis of auxin response factors (Glick *et al*., 2007 and Kang *et al*., 2010). Though many drought-tolerant cultivars have been released for cultivation by Tea Research Institutes and foliar applications of nutrients and PGRs are also recommended there are practical limitations.

The use of microbes is considered as the most promising drought ameliorative measure to protect the plants from abiotic stress (Sivakumar *et al.,* 2017) through the microbe mediated mitigation of drought stress conditions (Meena *et al.,* 2017). Introduction of Pink Pigmented Facultative

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Methylotrophs (PPFM) bacterial strains is one of the strategies used to mitigate the drought stress. This eco-friendly approach has been already established in the crops such as paddy, sunflower, soya bean, *etc*., Recently, Tamil Nadu Agricultural University, Coimbatore successfully demonstrated the technology in the paddy field where the crops remained greener even after 15 – 20 days of treatment (Saravanakumar *et al.,* 2014). Hence, the present study focused on isolation and *in vitro* screening of native isolates of PPFM in tea ecosystems of South India and also to assess its promotional ability through phytohormone secretion and ACC deaminase activity. This may overcome the drought stress for sustainable crop productivity in tea plantations.

Materials and methods

Survey

This investigation was conducted at the Parry Agro R&D Centre, Parry Agro Industries Ltd., Murugalli Estate, Valparai, Coimbatore Dist., Tamil Nadu, India. The area under study is situated between 8.0° and 13.0° N and 75.0° and 78.0° E, in the Western Ghats in South India. Initially, field surveys were carried out in all the tea-growing districts of south India *viz,* Anamallais, High Range, Central Travancore, the Nilgiris, Nilgiri-Wynaad, Wynaad, and Koppa for the isolation of PPFM (*Methylobacterium*) strains from the 62 leaf samples from both healthy (not affected by drought) and drought hit areas (both high temperature and water stress).

Isolation and *in vitro* screening of Methylobacterial strains (PPFMs)

Leaf samples collected from various experimental site, were subjected to isolation of PPFM by imprinting and serial dilution methods (Ram Kailash *et al*., 2010). The PPFM colonies were confirmed by the appearance of pink pigments when cultured on Ammonium Mineral Salt (AMS) agar media supplemented with 0.5% methanol and cycloheximide (Lidstrom and Chistoserdova, 2002; Whittenbury*et al*., 1970). Morphological, cultural, and biochemical tests were carried out adopting the standard bacteriological technique described in Ragavendra *et al*., (2019). The purified isolates were screened for phytohormone secretion and enzyme activities .

Estimation of IAA, GA_3, and Carotenoids produced by Methylobacterial strains (PPFMs)

The secretion of phytohormones was estimated by extracting the culture filtrate of PPFMs twice with an equal amount of ethyl Acetate. The quantum of IAA produced by the bacterial isolate was determined spectrophotometrically at 530 nm using Salkowski's reagent along with standard (Gordon & Weber (1951). Estimation of GA_3 and Carotenoids produced by the selected PPFM strains was determined using UV-Vis spectrophotometer-based analytical method described by Mahadevan and Sridhar, (1986).

Estimation of ACC deaminase produced by Methylobacterial strains (PPFMs)

All the PPFM isolates were grown in a DF mineral salt medium supplemented with 10 µm of ACC (Sigma - Aldrich, USA). ACC deaminase was quantified spectrophotometrically by following procedure enumerated by Honma and Shimomura (1978) and expressed as n moles of α–ketobutyrate produced per mg protein/h (Chinnadurai *et al*., 2009; Shaik Zulfikar Ali *et al.,*2013).

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and the significant means were segregated by critical difference (CD @P=0.05) at various levels of significance using the formulae given by Gomez and Gomez (1984).

Results and discussion

Tea growing areas in south India are located mostly in the Western Ghats with an elevation ranging from 500 to 2400 m above the MSL. The elevation is a remarkable factor that determines the temperature, rainfall pattern, humidity of south Indian tea cultivation with varied microbial

biodiversity. Evidences have started accumulating citing the importance of water availability for determining species microhabitat distributions (Webb and Peart 2000) annual rainfall and length of dry periods (Gentry 1988, Swaine and Becker 1999, Bongers and Ferris 1999). The microbial application is one of the most cost-effective and eco-friendly methods for the management of abiotic stresses in developing countries like India. Anitha *et al.,* (2010) stated about the isolation of PPFMs from the rhizosphere, endosphere, and phyllosphere layers in various mono-and dicotyledonous plants.

In the present investigation, field surveys were carried out in the major tea-growing areas of southern India (Tamil Nadu, Kerala, and Karnataka) for isolation and *in vitro* screening of PPFMs from phyllosphere layers. The pure colonies of PPFMs were cultured *in vitro* on AMS agar media and maintained at 4°C for the estimation of the phytohormonal secretion and ACC-deaminase enzymatic activities (Plate 1). Among 253 PPFM isolates, 119 isolates were obtained from leaves of tea bushes (not affected by high temperature) and 134 isolates from the leaves from drought-affected areas. The results confirmed the abundance of PPFMs in the phyllosphere of tea growing districts . This indicated that the PPFMs are indigenous and have a symbiotic association with the tea foliage. A higher number of isolates was recorded from the leaves of both the healthy tea bushes (not affected by high temperature) and drought-affected areas of Anamallais and Karnataka regions.

Plate 1. Isolation of *Methylobacterium* PPFMs from different agro-climatic zones of south India

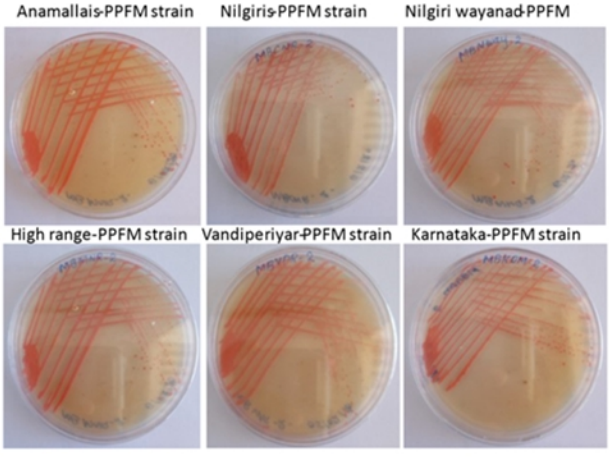

Table 1. PPFM isolates from phyllosphere of healthy and drought affected areas during drought season

S.No.	Location	No. of *Methylobacterium* isolates in	
		Phyllopshere in healthy area	Phyllopshere in drought area
1	Anamallais	18	42
2	High range	12	12
3	Central Travancore	15	15
4	The Nilgiris	9	16
5	Nilgiri Wayanad	12	5
6	Wayanad	14	13
7	Karnataka	39	31
	Total strains	**119**	**134**

Table 2: Plant growth promoting hormones (µg mL^{-1}) produced by the PFFM isolates

Regions	Isolates of Leaf samples from Drought affected area				Isolates of leaf samples from Healthy area			
	IAA	GA_3	Carotenoids	Mean*	IAA	GA_3	Carotenoids	Mean*
Anamallais	6.8 ± 0.007	44.9± 0.2	1.4±0.04	17.7± 0.08	20 ± 0.003	89 ± 0.2	1.5±0.02	36.8± 0.07
High Range	9 ± 0.041	52± 0.08	0.8±0.02	20.6± 0.05	17.9 ± 0.007	49.6 ± 0.01	1.6±0.04	23.0± 0.02
The Nilgiris	3.7 ± 0.002	60.6± 0.01	1.3±0.01	21.9± 0.01	2.1 ± 0.064	70.7 ± 0.13	1.1±0.07	24.6± 0.09
Nilgiri - Wayanad	2.1 ± 0.017	45± 0.01	0.9±0.09	16.0± 0.04	1.1 ± 0.014	63 ± 0.05	0.8±0.11	21.6± 0.06
Wayanad	7.5 ± 0.08	22.3± 0.07	1.5±0	10.4± 0.05	10.3 ± 0.002	37.9 ± 0.01	1.4±0.01	16.5± 0.01
Central Travancore	32.8 ± 0.003	93.1± 0.08	2.3±0.04	42.7± 0.04	26.4 ± 0.034	67.9 ± 0.03	1.1±0.02	31.8± 0.03
Karnataka	10.1 ± 0.031	35.6± 0.05	0.8±0.05	15.5± 0.04	30 ± 0.002	41 ± 0.02	2.4±0.04	24.5± 0.02

*Values represented are the means of five strains with three replicates in each region

The natural association of phyllosphere derived PPFMs have been reported in various agriculture crops. However, only a few reports are available focusing on the PPFMs in the tea ecosystem. Chinese researchers, Xie *et al.,* (2020) and Cakmakci (2019) reported the isolation approaches and the distribution of *Methylobacterium* in the acidic tea rhizosphere soil. In North India, Bora *et al.,* (2021) stated the distribution and the abundance of *Methylobacterium* in the tea endosphere layer. Plant growth-promoting rhizobacterial strains (PGPRs) are documented in the tea plantations of South India (Nepolean *et al.,* 2015). But the role of PPFMs and their bioactive compounds in the tea ecosystem remain unexplored so far. In this context, an extensive field survey was carried out and the effective strains of *Methylobacterium* were identified from Tea phyllosphere in South India (Table 1).

The present investigation demonstrated that there is a significant variation in the concentration of phytohormones (IAA, GA_3, and Carotenoids) and ACC deaminase enzyme activity in the PPFMs isolated from different agro-climatic zones of south India (Table 2 & 3) which is in the order of Central Travancore (Vandiperiyar) followed by Karnataka (Koppa) regions when compared to other mid and high elevation tea districts of Anamallais (Valparai) Nilgiris, High range (Munnar). Among the 253 PPFM isolates, 35 were identified based on the higher phytohormonal production region-wise (Table 4). Senthilkumar and Krishnamoorthy (2017) and Kumar *et al*., (2019) emphasized that among the phytohormones secreted by the methylotrophic bacteria, IAA, GA_3, Carotenoids influence plant growth, seed germination and help plants to endure water stress.

Phytohormones : Among 35 isolates tested, 5 strains *viz*., MBVPR19D.L23 (51.7 µg/mL), MBANML10H.L06 (45.1 µg/mL), MBVPR19D.L22 (42.0 µg/mL), MBANML10H.L11 (40.5 µg/mL) and MBVPR UPASI L.An H.L13 (40.0 µg/mL) were found to produce high level of IAA, GA_3, and Carotenoids.

IAA : A higher level of IAA production was observed in four isolates namely MBKDMV.G26H.L37 (42.1 µg/mL) followed by MBVPR19D.L22 (36.5 µg/mL), MBKDMK.T21H.L14 & MBVPR19D.L23(32 µg/mL).

GA_3 : Three efficient strains *viz.,* 1) MBVPR19D.L23, 2) MBANML10H.L06, 3) MBCNRLOC5D.L23 were found to secrete high concentrations of GA_3 when compared to other isolates.

Carotenoid : The concentration of carotenoid in all the tested isolates ranged from 0.5 – 3.09 µg/mL. The maximum levels were observed in three potential strains namely 1) MBVPR19D.L22, 2) MBKDMV.G26H.L37, and 3) MBKDMK.T21H.L14 which produced at the rate of 3.09 µg/mL, 2.78 µg/mL and 2.66 µg/mL, respectively. Based on the production potential of plant growth promoting hormones and ACC deaminase activity, three PPFM strains were identified from the drought-prone areas, Vandiperiyar *viz.,* MBVPR19D.L22 (Carotenoids), MBVPR19D.L23 (GA_3), and Karnataka viz., MBKDMV.G26H.L37 (IAA) regions (Table 4) for further study. Xiaomei Yan, *et al.,* (2018), reported that the endophytic *Methylobacteria* obtained from two cultivars showed remarkable plant growth-promoting abilities.

Table 3. Activity of ACC-*deaminase by* the PPFM isolates from leaf samples of drought affected and healthy area. (n moles of α-ketobutyrate produced mg/Protein/h)

Regions	Isolates of leaf samples from Drought affected area	Isolates of leaf samples from Healthy area
Anamallais	687.9± 0.074	689.1± 0.003
High Range	443.3± 0.093	896.7± 0.002
The Nilgiris	790.9± 0.003	603± 0.128
Nilgiri – Wayanad	380± 0.074	869.1± 0.05
Wayanad	757± 0.057	813.1± 0.001
Central Travancore	1066.8± 0.003	559.4± 0.034
Karnataka	744.7± 0.031	983.6± 0.002

Values represented are the means of five strains with three replicates in each region

Table 4. Production of phytohormones -IAA ,Carotenoids , GA_3 and *ACC-deaminase* activity by the PPFM isolates* in different tea districts of South India

S.No.	Strain No	IAA (µg/mL)	Carotenoid (µg/mL)	GA_3 (µg/mL)	Mean*	ACC n moles of α-ketobutyrate produced (mg/Protein/h)
1	MBANML10 H.L 06	21.09±0.002	1.80±0.012	112.3±0.01	45.1	276.5±0.006
2	MBANML10 H.L 11	22.13±0.005	1.34±0.023	98.1±0.009	40.5	952.2±0.001
3	MBANML10 H.L 12	16.7±0.002	1.30±0.012	56.47±0.022	24.8	838.6±0.001
4	MBANML 10 D.L 19	10.0±0.006	1.86±0.038	32.2±0.017	14.7	794.5±0.012
5	MBANML 10 D.L 32	7.84±0.014	1.143±0.071	31.1±0.007	13.4	723.1±0.009
6	MBMNR16PKD2H.L03	22.4±0.013	1.82±0.075	10.26±0.026	11.5	928.7±0.002
7	MBMNR16PKD1H.L01	17.15±0.005	1.69±0.026	82.26±0.008	33.7	882.6±0.003
8	MBMNR16PKD3H.L06	14.3±0.004	1.24±0.020	56.1±0.002	23.9	878.8±0.002
9	MBMNR16PKD3H.L05	13.53±0.003	1.15±0.015	61.2±0.007	25.3	880.3±0.003
10	MBMNR16PKD4H.L09	12.3±0.0011	1.22±0.058	45.2±0.009	19.6	785.7±0.002
11	MBCNR LOC1 H.L 01	4.78±0.002	1.66±0.009	98.23±0.003	34.9	700.8±0.004
12	MBCNRLOC3 D.L 17	3.34±0.001	1.47±0.003	41.3±0.005	15.4	786.6±0.005
13	MBCNRLOC3 D.L 18	4.28±0.003	1.26±0.018	28.26±0.008	11.3	875.1±0.001
14	MBCNRLOC5D.L 23	3.38±0.003	1.18±0.017	112.1±0.004	38.9	710.8±0.004
15	MBCNRLOC4 D.L 20	3.13±0.001	1.09±0.006	72.16±0.004	25.5	656.6±0.003
16	MBNWG D.L 17	4.29±0.043	1.5±0.233	56.4±0.012	20.7	951.0±0.001
17	MBNWG H.L 14	1.21±0.006	0.87±0.028	87.3±0.005	29.8	928.7±0.002
18	MBNWG D.L 27	1.06±0.003	0.75±0.015	12.3±0.016	4.7	925.5±0.001
19	MBNWG H.L 08	1.16±0.005	0.713±0.021	35.36±0.049	12.4	915.3±0.003
20	MBNWG D.L 16	1.02±0.005	0.54±0.021	66.2±0.006	22.6	866.4±0.006
21	MBWAY4H.L 07	11.6±0.002	1.95±0.015	23.3±0.012	12.3	947.8±0.004
22	MBWAY10D.L 18	11.3±0.001	1.65±0.007	20.4±0.034	11.1	923.2±0.002
23	MBWAY3H.L 05	9.7±0.003	1.22±0.015	18.3±0.022	9.7	927.5±0.002
24	MBWAY3H.L 06	9.44±0.001	1.16±0.007	72.1±0.007	27.6	857.4±0.002
25	MBWAY3H.L 03	8.60±0.003	1.13±0.019	73.43±0.012	27.7	814±0.001
26	MBVPR19 D.L 22	36.5±0.013	3.09±0.094	86.5±0.01	42.0	1196.3±0.002
27	MBVPR19 D.L 23	32.05±0.006	1.91±0.035	121.1±0.035	51.7	1076.4±0.005
28	MBVPR UPASI L.A H.L 13	31.7±0.001	1.97±0.005	86.21±0.006	40.0	978.4±0.002
29	MBVPR19 D.L 24	29.8±0.003	1.74±0.020	70.92±0.023	34.2	927.7±0.001
30	MBVPR UPASI L.A D.L 28	29.6±0.013	1.26±0.070	56.2±0.007	29.0	861.6±0.003
31	MBKDM V.G 26 H.L 37	42.1±0.005	2.78±0.035	40.3±0.013	28.4	1045.9±0.002
32	MBKDM K.T 21 H.L 14	32.5±0.009	2.66±0.063	42.28±0.013	25.8	976.5±0.003
33	MBKDM M.G 6 D.L -40	28.8±0.008	1.94±0.048	41.17±0.007	24.0	957.4±0.004
34	MBKDM K.T 21 H.L 18	15.4±0.005	1.86±0.029	40.40±0.022	19.2	928.5±0.001
35	MBKDM16 H.L 13	14.7±0.051	1.57±0.283	44.23±0.011	20.2	905.2±0.003
	C.D.	0.058	0.796	0.254	0.4	0.011
	SE(m)	0.02	0.282	0.09	0.1	0.004
	SE(d)	0.029	0.398	0.127	0.2	0.005
	C.V.	2.239	13.156	2.098	5.8	0.023

*Values are the mean of the total phytohormone production of IAA, GA_3 and Carotenoids by the selected isolates

Phytohormones produced by PPFMs from healthy and drought affected areas

Production of growth hormones *i.e.* IAA, GA_3, Carotenoids in the isolates of leaves from healthy bushes is higher than that of drought affected area except Central Travancore. The highest ACC deaminase activity was registered in the isolates of Central Travancore namely MBVPR19D.L22 -(1196 n moles of **α**-ketobutyrate produced /mg protein/h) and MBVPR19D.L23-(1076 n moles of **α**-ketobutyrate produced /mg protein/h) (Table 2 & 3). Isolates obtained from Central Travancore registered high level of ACC deaminase activity (1066.8 n moles of **α**-ketobutyrate produced /mg protein/h) as compared to other regions.

Conclusion

The present investigation is mainly focused on the isolation and *in vitro* screening of effective *Methylobacterium* (PPFMs) for the increased production of phytohormones and enzymatic reactions. Based on the results of phytohormones and ACC deaminase activity, two isolates of Central Travancore (MBVPR19 D.L 22 and MBVPR19 D.L 23) and one strain from Karnataka (MBKDMV.G26H.L37) were selected for field application to mitigate the drought impact in tea plants by enhancing the phytohormonal production. The selected strains of MBVPR19 D.L 22 were identified as *Methylobacterium radiotolerans* (OL440712) using 16sRNA technique. The present study indicated that plant growth hormones and ACC deaminase enzyme activity secreted by *Methylobacterium* species could play critical role to mitigate the drought stress for a sustainable productivity in tea industry.

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