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Drought tolerant *Methylobacterium populi (*Nel-c) enhanced growth and disease resistance in amaranth

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ABSTRACT

Pink Pigmented Facultative Methylotrophs (PPFM) is important for growth promotion and stress tolerance in plants and can also stimulate induced systemic resistance against plant pathogens. PPFMs were isolated from amaranth leaves of ten major locations of amaranth in Thrissur district of Kerala. Under *in-vitro*, Seven PPFM isolates were positive for IAA production with highest production by Kod-c isolate (15.03 µg ml^{-1}).The isolate Pat-c showed highest nitrogen fixation of 0.766 mg g^{-1}. The amount of phosphate solubilization ranged from 5.99 to 11.15%. Eight isolates were positive for ACC deaminase activity. Three isolates (Kod-c: 62.7%, Kan a: 45.7% and Nel-c: 48.9%) inhibited *Rhizoctonia solani*, out of 10 isolates. Eight PPFM isolates were positive for ammonia production and none produced hydrogen cyanide and siderophore under *in-vitro* conditions. Nel-c PPFM isolate was tolerant to pH 3.5 and Nel-c and Kan-a isolates were tolerant to 20% PEG induced water stress. The three most promising PPFM isolates were identified as *Methylobacterium* sp. (Kod-c and Kan- a) and *M. populi* (Nel-c) using 16S rRNA sequencing. The three PPFM isolates significantly increased plant height, number of leaves, days taken for first flowering and weight of fresh shoots when compared to uninoculated plants. Disease severity was also lowest (54.08%) in the case of *M. populi* (Nel-c). The present study revealed that *M. populi* (Nel-c) isolate was the most promising stress tolerant native PPFM isolate for growth promotion and disease management in amaranth.

Key words: Methylotrophs, isolates, N_2 fixation, K solubilizer, disease severity.

INTRODUCTION

Pink-pigmented facultative methylotrophic bacteria (PPFMs) have been extensively studied for their ability to promote plant growth through various mechanisms. Key modes of action include the indole-3-acetic acid (IAA), cytokinins, gibberellin (GA), and 1-aminocyclopropane-1-carboxylate deaminase (ACC) production, nitrogen fixation and phosphorus solubilisation. PPFM also enhances plant stress tolerance and improves catalase enzyme activity, which helps the crop protect plants from various stresses (Nysanth *et al*., 9). These mechanisms, individually or in combination, positively influence the growth and development of host plants. Additionally, PPFMs have biocontrol capabilities that help mitigate the negative effects of various phytopathogens. Among methylotrophic organisms, facultative methylotrophic (FM) bacteria, particularly those belonging to the genus *Methylobacterium* and *Methylorubrum*, have been widely studied. These bacteria exhibit unique physiological characteristics and are found throughout various parts of plants, including leaf surfaces, stems, flowers, and roots. Furthermore, *Methylobacterium* sp. exhibit significant biocontrol activity against phytopathogens, protecting plants from harmful pathogens and thereby improving plant health. Seed treatment or foliar spray of *Methylobacterium* on rice induced the pathogenesis related proteins which protected the plants against sheath blight pathogen *Rhizoctonia solani* under pot culture conditions (Madhaiyan *et al*., 6)

Amaranth is a popular leafy vegetable of Kerala. The leaves and stems are good sources of calcium, vitamin A, iron, and vitamin C. The grain amaranth is also a rich source of protein and essential amino acids like lysine, leucine, isoleucine *etc*. Methylotrophic bacteria can help in both plant growth promotion, disease management, and drought mitigation in plants. The hypothesis is that the amaranth is a leafy vegetable, which is highly susceptible to drought and diseases. At present, there are no drought tolerant PPFM inoculants that can enhance the plant growth and manage the disease in amaranth. Since PPFMs can both promote plant growth and prevent infections by phytopathogens, they serve as promising alternatives to chemical fertilizers and fungicides in sustainable agriculture. There are very few studies on native stress-tolerant PPFM as bioagents in Kerala. Hence, the present study was undertaken with an aim to identify stress tolerant PPFM, which will help to promote the growth and manage the foliar disease in amaranth.

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MATERIALS AND METHODS

The samples of amaranth leaves were collected, after a survey, from ten major growing areas of Thrisur district in Kerala. Global Positioning System (GPS) coordinates of the locations were recorded (Table 1; Fig. 1). The Ammonium Mineral Salts (AMS) medium (Whittenburry *et al*., 20) was used for the isolation of methylotrophs. Upper and lower surface of fully grown samples of leaf were imprinted on the solidified AMS agar medium separately.

The predominant isolates from each location were assessed for growth promoting traits such as IAA production, nitrogen fixing ability, phosphate solubilizing ability (Nguyen *et al*., 8), potash solubilization (Panhwar *et al*., 10), ACC deaminase enzyme production, ammonia production (Cappucino *et al*. 1), HCN production and siderophore production

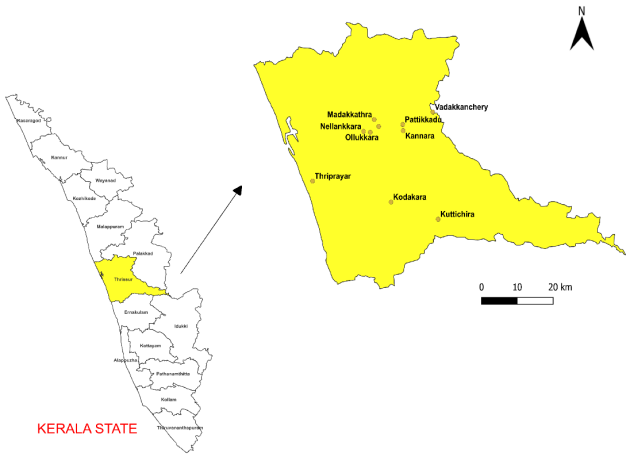

Fig. 1. Leaf samples of amaranth collected from different locations of Thrissur district in Kerala.

Table 1. Location of the leaf samples collected from major amaranth growing areas of Thrissur district in Kerala.

Location	Isolate code	Latitude ^{0}N	Longitude ^{0}N	Elevation (Mean sea level)
Vellanikkara	Vel	10.5452 N	76.2740 E	22 m
Nellankkara	Nel	10.5339 N	76.2349 E	6 m
Thriprayar	Trp	10.4203 N	76.1049 E	7 m
Kodakara	Kod	10.3723 N	76.3053 E	19 m
Madakkathra	Mad	10.5612 N	76.2624 E	75 m
Kannara	Kan	10.5356 N	76.3362 E	55 m
Vadakkanchery	Vad	10.6008 N	76.4904 E	38 m
Kuttichira	Kut	10.3329 N	76.4256 E	64 m
Pattikkadu	Pat	10.5496 N	76.3352 E	87 m
Ollukkara	Oll	10.5319 N	76.2523 E	6 m

(Schwyn and Neilands, 17) under *in vitro*. All the predominant isolates were subjected to antagonism against *Rhizoctonia solani* (Skidmore and Dickinson, 19). The PPFM isolates were also assessed for drought tolerance.

The three most efficient PPFM isolates were identified and confirmed using the 16S rRNA sequencing. DNA was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. PCR amplification reactions were carried out in a 20 µl reaction volume, which contained 1X PCR buffer (100 mM Tris HCl, pH-8.3; 500 mM KCl), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 mM $MgCl_2$, 1 unit of Ampli*Taq* Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5 pM of forward and reverse primers and template DNA. PCR products were analyzed on 1.2% agarose gels prepared in 1X TAE buffer containing 0.5 µg/ml ethidium bromide. A BLAST search was done to determine the nucleotide sequence and nucleotide homology of the isolates. NCBI offers BLASTn tool. The phylogenetic tree of selected PPFM isolates were performed using MEGA7 sequencing of 16S rRNA genes.

Pot culture study was conducted to evaluate the effect of three most potential PPFM (Kod-c, Nel-c and Kan-a) isolates for yield improvement and drought tolerance in amaranth at Kerala Agriculture University from May, 2021 to October 2021.

Healthy seeds (100 g) of amaranth variety "Arun" were soaked in 10 ml of PPFM culture broth containing 10^9 CFU ml^{-1} for 20 min and then air dried, after which seeds were dibbled in portrays. Coir pith compost was sterilized by autoclaving at 121°C for 60 min for three consecutive days. Coir pith (sterilized) was added into potrays containing 3.2 cm diameter well. Amaranth seeds were sterilized using sodium hypochlorite (1%) for 3 min under aseptic environment. The seeds were washed in sterile water for 3-4 times. The surface sterilized seeds of amaranth were placed in portrays and were irrigated with sterile water. Twenty-one-days-old seedlings were used with one seedling per well. Sterile water was used for irrigation.

The potting mixture consisted of soil, sand and farmyard manure (1:1:1) and was sterilized for three consecutive days. The sterilized potting mixture was filled into grow bag of dimension (23 × 28 cm). PPFM inoculum was prepared by inoculating 72 h cultures in AMS broth. The flasks were incubated at 28°C for five days in order to obtain a population of 10^9 CFU ml^{-1}. Sterilized talc powder was mixed with the PPFM broth culture in 1:3 ratio to obtain the PPFM inoculant.

The following efficient PPFM bacterial isolates *viz*., PPFM-1 (Kod-c), PPFM-2 (Nel-c) and PPFM-3 (Kan-a) were evaluated under pot culture and TNAU reference strain was used for comparison. Seven treatments with 10 replications (T1- PPFM-1 (Kod-c), T2-PPFM-2 (Nel-c), T3- PPFM-3 (Kan-a), T4- Reference PPFM strain, T5- Organic Package of Practice of (KAU), T6- Package of Practices (KAU) and T7- Control) were used.

Leaf surface of amaranth was sprayed with potential native PPFM isolates. The PPFM cultures were multiplied for 5 days (10^9 CFU ml^{-1}). It was diluted in 1:1 ratio with sterilized distilled water and sprayed @ 0.5 ml per plant on the amaranth leaves. Challenge inoculation of *R. solani* in amaranth plant was done at 30 days after planting (DAP) using mycelia and spores suspension (2×10^4 conidia ml^{-1}) mixed with sterilized distilled water. One mililitre suspension of *R. solani* was placed directly on the needle-punched wound made on amaranth leaves. In order to prevent the loss of moisture after inoculation, plastic bags were used to cover the seedlings for 2 days. Plant height, number of leaves, and days taken for first flowering were recorded. Disease severity was determined by recording the scores taken every 10 days interval using 0-9 grade scale (Nair and Anith, 7).

RESULTS AND DISCUSSION

The microbial population on leaf samples from adaxial surfaces ranged between 1.69 to 8.98 cfu cm^{-2} and abaxial surfaces from 1.60 to 10.18 CFU cm^{-2} (Table 2). The isolate Vel (Vellanikkara) sample recorded a significantly higher PPFM population (8.98 CFU cm^{-2}) on its adaxial surface followed by Mad (5.53 CFU cm^{-2}). Abaxial surface recorded higher population in the case of Vel (10.18 CFU cm^{-2}) and showed significantly higher population followed by Mad (5.96 CFU cm^{-2}).

Dhanalakshmi (3) documented that population of PPFM in adaxial and abaxial surfaces of mulberry leaves ranged between 9 to 25 CFU cm^{-2} and 11 to 24 CFU cm^{-2}, respectively. The population of PPFM in a particular environment is affected by both biotic and abiotic factor. The influence of multiple climate factors could have result in a lesser population of PPFMs on the adaxial surfaces.

The results were compared with the standard keys for PPFM identification (Corpe and Rheem, 2) and the isolates were tentatively identified as PPFM. IAA production was 3.15-15.03 µg ml^{-1} (Table 3). IAA production by Kod-c (15.03 µg ml^{-1}) was significantly higher followed by Nel-c (9.60 µg ml^{-1}), and Kan-a (9.42 µg ml^{-1}), which were on par with each other. Santosh *et al.* (16), reported that IAA production and secretion by different strains of *Methylobacterium* ranged from 1.27 to 19.77 µg ml^{-1}, which is in accordance with the present studies. The results prove that PPFM produces IAA, which are known to regulate cell division, cell elongation and increase root hair formation.

Table 2. Population of phylloplane PPFM from major amaranth growing areas of Thrissur district.

Location	Isolate code	Population of PPFM (CFU cm^{-2})	
		Adaxial surface	Abaxial surface
Vellanikkara	Vel	8.98 (0.95)[a]	10.18 (1.00)[a]
Nellankkara	Nel	4.92 (0.69)[c]	5.73 (0.75)[bc]
Thriprayar	Trp	4.67 (0.67)[c]	5.10 (0.70)[d]
Kodagara	Kod	4.89 (0.68)[c]	5.38 (0.72)[cd]
Madakkathra,	Mad	5.53 (0.74)[b]	5.96 (0.77)[b]
Kannara	Kan	4.78 (0.68)[c]	4.37 (0.64)[e]
Vadakkanchery	Vad	1.94 (0.28)[d]	2.70 (0.43)[f]
Kuttichira	Kut	1.96 (0.29)[d]	2.33 (0.36)[g]
Pattikkadu	Pat	1.69 (0.22)[e]	1.60 (0.20)[h]
Ollukkara	Oll	1.99 (0.29)[d]	2.33 (0.36)[g]
$CD_{(0.05)}$		0.269	0.341

Log transformed values are given in parantheses.

PPFM (Pat-c) isolate recorded significantly higher nitrogen fixation (0.766 mg g^{-1}) and this was on par with Nel-c (0.756 mg g^{-1}) (Table 3). Raghavendra and Santhosh (13) also reported that the PPFM fixes nitrogen in the range of 0.39 to 1.32 mg g^{-1}, which is in agreement with the current results. Highest phosphate solubilization was in the case of Nel-c (11.15%), which was on par with TNAU reference strain (10.93%) followed by Kan-a (10.42%). Similar results were obtained by Sheela *et al.* (18), who documented phosphate release from 7.17 to 9.80% in *Coleus forskohlii*.

The amount of potassium solubilization was highest in the case of Kan-a (8.40 µg ml^{-1}) followed by Kod-c (8.19 µg ml^{-1}) (Table 3). Six isolates exhibited clear solubilization zones around colonies (Vel-a, Nel- Mad-a c, Kod-c, Kan-a and Kut-c).Similar results were observed by Prajapati *et al.* (12) for strains of M-10, M-15, M-8, M-29 potassium solubilizers on Alexandrov's medium. In the present investigation, the amount of potassium solubilized was highest in Kan-a (8.40 µg ml^{-1}) followed by Kod-c (8.19 µg ml^{-1}) and Kut-c (7.87 µg ml^{-1}).

Among the isolates tested, there was only one isolate (Nel-c) that showed highest ammonia production. Three isolates (Kan- a, Pat-c, Oll-d) were categorized as moderate and the reference strain also exhibited moderate production. Four isolates were found as weak producers (Vel-a, Trp-b, Kod-c, Kut-c) and two PPFM isolates (Mad-a, Vad-b) showed

Table 3. *In-vitro* screening of PPFM isolates for plant growth promoting traits.

Isolate	Concentration of IAA (µg ml^{-1})	Nitrogen fixation (mg g^{-1} of malate)	Pi-release (%)	Quantity of K solubilized (µg ml^{-1})	ACC deaminase activity
Vel-a	4.26 ^{e}	0.396^{f}	7.73^{e}	6.50^{e}	+
Nel-c	9.60 ^{b}	0.756^{ab}	11.15^{a}	7.14^{d}	++
Trp-b	8.28^{c}	0.366^{f}	6.20^{g}	-	++
Kod-c	15.03^{a}	0.583^{d}	10.01^{c}	8.19^{b}	+++
Mad-a	-	0.480^{e}	7.49^{e}	6.27^{f}	+
Kan-a	9.42^{b}	0.656^{c}	10.42^{b}	8.40^{a}	++
Vad-b	-	0.480^{e}	7.44^{e}	-	++
Kut-c	-	0.696^{bc}	5.99^{g}	7.87^{c}	++
Pat-c	3.15^{f}	0.766^{a}	7.11^{f}	-	-
Oll-d	8.01^{cd}	-	8.94^{d}	-	-
Reference PPFM (TNAU)	7.55^{d}	0.350^{fg}	10.93^{a}	-	+++
$CD_{(0.05)}$	0.566	0.066	0.315	0.041	-

Vel-a: Vellanikkara, Nel-c : Nellankkara, Trp-b : Thriprayar, Kod-c: Kodagara, Mad-a : Madakkathra, Kan-a :Kannara, Vad-b : Vadakkanchery, Kut-c: Kuttichira,Pat-c: Pattikkadu, Oll-d: Ollukkara, Reference PPFM: TNAU reference culture

no ammonia production. None of the PPFM isolates produced siderophore and HCN production under *in vitro* conditions. Poorniammal *et al*. (11) also reported that all *Methylobacterium* strains recorded negative reaction to HCN production, which agrees with the present studies.

Three (Kod-c, Kan-a, Nel-c) out of ten PPFM isolates inhibited *R. solani* and Kod-c isolate showed the highest zone of inhibition (60.9%) followed by Kan-a (45.4%). Poorniammal *et al*. (11) reported that PPFM inhibited the mycelial development of *Sclerotium rolfsii*, *Pythium*, *Fusarium oxysporum*, *Fusarium udum*, *Macrpohomina*, *Phytophthora* and *R. solani. R. solani* incidence, which cause leaf blight in amaranthus is severe during rainy season. Similar observations were made by Kassem *et al*. (5) also, reported highest inhibition of mycelial growth by *Methylobacterium* sp. and *Methylobacterium rhodinum* recorded were 72.61 and 69.30%, respectively, which agrees with the present studies.

Among the PPFM isolates, Nel-c isolate had the highest OD of 0.89 and 0.32 at 10 and 20% water stress respectively and found that Nel-c isolate was tolerant to acidic pH up to 3.5. Acid tolerant methylotrophic microbes isolated from native soil are ideal for use as biofertilizers and biocontrol agents in crops grown in acidic soils of Kerala. PPFM isolates were ranked according to their growth promoting characteristics, antagonistic activity against *R. solani* and its mechanisms, drought, and acidic pH tolerance. The isolates Kod-c, Nel-c and Kan-a were selected as the three most efficient PPFM isolates based on all the parameters tested.

The three most promising PPFM isolates were further identified and confirmed based on the 16S rRNA sequencing. Analysis of sequence data for isolate Kan-a showed 94 per cent query coverage and 100 per cent identity to *Methylobacterium* sp. (Genebank Accession No. MN982830.1). The isolate Nel-c showed 89 per cent query coverage and 96.49 per cent identity with *Methylobacterium populi* (Gene bank Accession No**.** AB698694.1) and the isolate Kod-c showed 90 per cent query coverage and 99.70 per cent identity with *Methylobacterium* sp. (Genebank Accession No. MH769104.1). Identified isolates and their sequences have been deposited in the NCBI GenBank.

Considering the plant growth promoting and antagonistic traits of different isolates, *Methylobacterium* sp. (Kan-a), *Methylobacterium populi* (Nel-c) and *Methylobacterium* sp. (Kod-a) were evaluated under pot culture studies. Inoculation of the *Methylobacterium* strain showed significant differences with respect to plant growth parameters of amaranth. Significant increase in the plant height, number of leaves and days taken for first flowering were noticed at various growth stages of the crop as influenced by inoculation of PPFM. *Methylobacterium populi* (Nel-c T_2) recorded significantly highest plant height, the number of leaves, and the fresh weight of yield at harvests .Days for first flowering was early in Nel-c (T_2-PPFM-2). *Methylobacterium populi* (Nel-c T_2) treatment showed highest fresh weight harvest (61.66 g $plant^{-1}$) (Table 4). *Methylobacterium* applied to leaves significantly increased plant height, leaf area index, number of mature pods and harvest index of

Table 4. Effect of PPFM on biometric parameters and disease severity at 90 DAP.

Treatment	Plant height (cm)	No. of leaves	Days taken for first flowering	Total harvest (g plant^{-1})	Disease severity (%)
T1 : PPFM-1	92.0^{b}	78.4^{b}	83	57.30^{b}	56.13^{e}
T2 : PPFM-2	94.53^{a}	80^{a}	82	61.66^{a}	54.08^{f}
T3 : PPFM-3	88.68^{c}	76.6^{c}	83	53.92^{c}	56.04^{e}
T4 : Reference culture of PPFM (TNAU)	86.84^{d}	73.9^{d}	85	49.85^{d}	58.29^{d}
T5 : Organic package of practices of KAU	85.52^{e}	72.2^{e}	83	45.96^{e}	63.82^{c}
T6 : Package of practices of KAU	83.95^{f}	70.7^{f}	85	42.06^{f}	65.32^{b}
T7 : Control	81.65^{g}	68.3^{g}	86	37.73^{g}	69.29^{a}
$CD_{(0.05)}$	0.835	1.42	NS	1.10	1.13

DAP: Days after planting; PPFM-1 : Kan-a (*Methylobacterium* sp.); PPFM-2 : Nel-c (*Methylobacterium populi*); PPFM-3 : Kod-c (*Methylobacterium* sp.).

peanut (Gashti *et al.*, 3) which is in agreement with the present studies. Raja and Sundaram (14) also reported that PPFM inoculation increased cotton dry matter production, seedling vigor and yield under field conditions. PPFM isolates, when applied on leaves significantly increased antioxidant enzymes, growth and yield of snap bean plants under field conditions (Gawad *et al*., 4), which is in agreement with the present studies.

Disease severity was recorded (Table 4) at 90 DAP and *Methylobacterium populi* (Nel-c isolate) (T_2) showed lowest disease severity. It was a highly effective biocontrol agent for controlling *R. solani* in amaranth. It is in agreement with the Santosh and Sreenivasa (15), who reported higher population of *M. populi* in chilli phyllosphere and rhizosphere. The experiments revealed the potential use of PPFM strains as a prophylactic measure against foliar diseases. *Methylobacterium* strain have the ability to improve plant growth and to induce plant defense agents against *R. solani*. Previous investigations confirm that the methylotrophs inhibits foliar pathogens from growing on phyllosphere. *Methylobacterium* sp. are known for producing growth regulators and they are also capable of controlling the *R. solani*.

Based on the present study, *Methylobacterium populi* (Nel-c) was the most promising drought-tolerant isolate for plant growth promotion and *Rhizoctonia solani* management. It was the highest producer of IAA (9.60 µg ml^{-1}), nitrogen fixing ability (0.756 mg g^{-1} malate), phosphate solubilization (11%), K-solubilization (7.14 µg ml^{-1}) and lowest disease severity under *in-vitro* and pot culture studies. By using native PPFM isolates, both, growth promotion and disease management in amaranth can be achieved in an eco-friendly manner. However, further studies are required under field conditions to confirm the efficiency of the PPFM isolates.

AUTHORS' CONTRIBUTION

Conceptualization of research *(AC, KSG);* Analysis of data and interpretation *(AC, KSG);* Preparation of the manuscript (DJ, DJ, ST).

DECLARATION

There are no conflict and competing interest among the authors.

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