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RESEARCH ARTICLE

# Pink Pigmented Facultative Methylotrophs (PPFMs) improve rooting in black pepper (*Piper nigrum* L.) cuttings and mitigate drought stress

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## Abstract

Black pepper, a major spice cultivated across the globe, is drought-sensitive, and water stress often results in plant fatality. Pink Pigmented Facultative Methylotrophs (PPFMs), the plant growth-promoting phyllosphere bacteria, can induce physiological changes in plants, making them more tolerant to various stresses. PPFMs from phyllosphere of black pepper were isolated, characterized and screened for plant growth promotion and drought stress mitigation in black pepper. Sixty PPFM isolates were obtained by leaf imprinting and plating on 5 % PEG 6000 amended Methanol Mineral Salts medium. Screening of isolates for plant growth promotion was based on producing indole acetic acid, gibberellic acid, extracellular ammonia and 1-aminocyclopropane-1-carboxylate deaminase activity *in vitro*. The influence of three best performing isolates, identified by 16S rRNA sequencing as *Methylobacterium aerolatum* PNPPFM60, *Methylorubrum aminovorans* PNPPFM59 and *Methylorubrum zatmanii* PNPPFM44, on growth and establishment of black pepper cuttings was investigated. *M. aerolatum* PNPPFM60 recorded the highest biometric parameters. After the foliar application on 90$^{th}$ DAP the soil moisture level was maintained at field capacity for 15 days and then irrigation was withdrawn. The control plants showed symptoms of drought after 3 days of withdrawing irrigation while the PPFM treated plants remained green with higher RWC and higher cell membrane integrity till 7$^{th}$ day. The antioxidant enzymes were highest in plants treated with *M. aerolatum* PNPPFM60. PPFMs could improve growth of black pepper and mitigate drought stress in early stages of establishment of black pepper and has the potential to be developed as a bioformulation.

## Keywords

ACC deaminase; extra cellular ammonia; GA; IAA; *Methylobacterium aerolatum*

## Introduction

Black pepper (*Piper nigrum* L.), the "King of Spices" is a flowering vine from the family Piperaceae and is cultivated primarily for its fruit. The inflorescence is a catkin-like spike with small, white to greenish-white flowers which develops into drupe (single-seeded berries), which are dried and used as a spice and seasoning. Vietnam leads the world in black pepper production, producing 272,235 tons in 2022 accounting for over one-third of the global supply. This is more than double the output of Brazil, which holds second place with 128,331 tons followed by Indonesia with 81,962 tons,

Burkina Faso at 76,856 tons and India at 64,205 tons, completing the top five producers (1). Kerala and Karnataka account for a major portion of production of black pepper in India.

Research on the impact of climate change on black pepper production has predominantly concentrated on the effects of temperature and precipitation. Black pepper is susceptible to water stress, with numerous studies indicating a decline in pepper yield when exposed to water-deficient conditions (2). Extended growing seasons alter evapotranspiration rates, thereby impacting water demand in managed ecosystems. Key growth phases, such as flowering and pollination, are particularly vulnerable to weather conditions and are crucial for determining final yields (2). With rising global temperatures, the likelihood of crop failures in traditional production regions increases, especially if climate variability intensifies and precipitation decreases or becomes more erratic. The productivity of black pepper in India has started to decline in recent years due to the poor yield of the varieties cultivated, lack of scientific cultivation and the occurrence of crop loss from diseases, pests, and drought (3). Subsequently, research focusing on effective, low-cost and eco-friendly technology and use of microorganisms gained importance to help the plants withstand abiotic and biotic stresses. Understanding how microbes facilitate morphological, physiological, and genetic changes in plants to cope with climate change can significantly boost crop production.

Recent research has revealed that specific microorganisms can enhance the potential of plants to withstand drought stress. Among these, a group of facultative methylotrophic bacteria known as Pink Pigmented Facultative Methylotrophs (PPFMs) is notable for its capacity to colonize plant surfaces and promote growth (4). These bacteria are strictly aerobic, Gram-negative and exhibit a rod-shaped morphology. They are capable of metabolizing single carbon compounds, including methane, methanol and methylamine, with some also utilizing C2, C3 and C4 carbon sources. PPFMs are classified as α-Proteobacteria and are commonly found in association with various crop species (5).

Methanol, which is abundant in plant tissues, serves as a crucial carbon source for these methylotrophs, enhancing their ability to colonize the phyllosphere (4). PPFMs have the potential to significantly boost plant growth and mitigate the stressors related to climate change. Investigating the relationships between these beneficial bacteria and plants under varying biotic and abiotic stresses could yield valuable insights for developing effective bio-inoculants to improve plant health and productivity. PPFMs promote plant growth through the production of growth hormones like indole acetic acid and phosphorus solubilisation and several other mechanisms (6, 7). They can also modify agronomic characteristics like shoot length, root length and seedling vigour, as well as heat or cold tolerance (8). PPFMs hold promise as effective agents for enhancing the plant's water balance, improving photosynthetic rates, and promoting the synthesis of compatible osmolytes such as proline. Furthermore, these microorganisms increase the activity of antioxidant enzymes like catalase and peroxidase, contributing to enhanced plant stress tolerance (9).

Considering the plant growth promoting and stress mitigating potential of the PPFMs a study on the isolation, screening, and characterization of PPFMs from the leaves and rootzone of black pepper was conducted to assess their effect on plant growth and drought tolerance in black pepper cuttings.

## Materials and Methods

### *Isolation and characterisation of PPFMs*

Leaf samples (3-5 leaves) from healthy black pepper plants and 50 g of soil from the rhizosphere were collected from various regions in the southern part of Kerala, India. These samples were placed in sterile polythene bags and stored at 4 °C for further studies. Isolation of moisture stress tolerant PPFMs was done on modified Ammonium Mineral Salts (AMS) medium containing 0.5 % methanol (10) enriched with 5 % Polyethylene Glycol (PEG) 6000. Phyllosphere methylotrophs were isolated using the leaf imprint technique (Fig. 1), while rhizosphere methylotrophs were obtained through serial dilution and plating (11). Distinct, pink-pigmented colonies were preserved on peptone glycerol agar slants at 4 °C for future experimentation. Characterization of the pink isolates included assessments of colony size, shape and colour, as well as Gram staining and oxidase and catalase tests (12).

### *Assessment of plant growth promoting traits*

The potential of PPFM isolates to produce Indole Acetic acid (IAA), Gibberellic acid (GA), extracellular ammonia and 1-aminocyclopropane-1-caroxylic acid deaminase (ACCD) activity was assessed *in vitro*.

### *Quantitative estimation of Indole Acetic Acid and Gibberellic acid production*

IAA production by the selected isolates was estimated in AMS broth amended with 0.1 % tryptophan as well as without amendment. The flasks after inoculation of the log phase isolates were placed for incubation at 28 ± 2 °C under shaking conditions for 7 days. Then the cells were pelleted at 10,000 rpm for 10 min at 4 °C in refrigerated centrifuge and supernatant was used for IAA and GA estimation. The estimation of IAA was done by colorimetric method using Salkowski reagent in a UV-Vis spectrophotometer (Shimadzu UV-1900 i) at 530 nm (13). Gibberellic acid was extracted from the supernatant of the selected bacterial isolates using standard procedure and the estimation of GA was carried out spectrophotometrically at 254 nm (14).

### *Quantitative Estimation of Extracellular Ammonia Production*

Extracellular Ammonia production was estimated by Nessler's reagent method. The selected isolates grown in ten mL peptone broth under shaking conditions for 72 hr were subjected to centrifugation at 4 °C for 15 min at 4500 rpm. Development of brown colour in the supernatant on addition of 5 mL Nessler's reagent indicated ammonia pro-

**Fig. 1.** Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs) from black pepper by leaf imprint method.

duction and was measured at 450 nm in UV-VIS spectrophotometer (Shimadzu UV-1900 i) and expressed as µmol $mL^{-1}$ of ammonium sulphate (12).

### *Quantitative Estimation of ACC deaminase activity*

ACCD activity of the isolates was estimated by growing the isolates on Dworkin and Foster (DF) minimal salt medium with ACC as the sole nitrogen source. The extraction was carried out using standard procedure and the absorbance of the supernatant was measured at 540 nm spectrophotometrically (Shimadzu UV-1900 i) and expressed as µmoles of α-keto butyrate produced (15).

### *Assessment of drought tolerance potential of the selected isolates in vitro*

The selected isolates were inoculated to 50 mL AMS broth supplemented with 0 %, 5 %, 10 %, 20 %, 30 % and 40 % w/v of polyethylene glycol 6000 (PEG 6000) (16). The flasks were incubated at 28 ± 2 °C in an orbital shaker at 120 rpm for 7 days. The bacterial growth was estimated by measuring the absorbance at 600 nm in a DEN-600, Photometer (Biosan Model No BS-050109-AAA) (17).

A weighted average ranking was employed to identify the most effective PNPPFM isolates with the greatest potential for promoting plant growth *in vitro*. The criteria for ranking included the production of IAA, GA, extracellular ammonia, ACC deaminase activity and drought tolerance. The top 3 isolates *viz.* PNPPFM 60, PNPPFM 59 and PNPPFM 44 were chosen as the most promising candidates based on this ranking method.

### *Molecular identification of the selected isolates*

16S rRNA sequencing was used for the molecular identification of the bacterial isolates. The PCR amplification with 16S rRNA universal primers (Forward primer - 16S-RS-F-CAGGCCTAACACATGCAAGTC and reverse primer 16S-RS-R-GGGCGGWGTGTACAAGGC was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems). PCR amplification profile was 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 40 s and 72 °C for 60 s followed by a final extension of 72 °C for 7 min for amplification of 16S rRNA genes. The PCR product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems) using Sanger DNA sequencing method. Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (18). Sequence similarity was checked using Basic Local Alignment Tool (BLAST) The contigs were compared against the sequences of 16S rRNA of bacterial isolates available in the National Centre for Biotechnology Information (NCBI) Nucleotide Database. The selected bacterial isolates were identified based on maximum percentage of similarity of the sequences.

### *Effects of selected PNPPFM isolates on plant growth promotion of black pepper cuttings*

The top three isolates obtained from the weighted average ranking were used for a pot culture experiment with 3 noded cuttings of black pepper *var*. Karimunda. The isolate (PPFM 38) obtained from the Department of Microbiology was used as the reference culture. The pot culture experiment with 7 treatments and 3 replications was laid out in Completely Randomized Design (CRD). The treatments were as follows: T1 – PNPPFM 60, T2 – PNPPFM 59, T3 – PNPPFM 44, T4 – PPFM 38 (Ref. culture), T5 – Sterile water, T6 – Uninoculated media and T7 – Absolute control. Ten cuttings were maintained per replication.

The PNPPFM isolates were grown in AMS media amended with 0.5 % methanol for 7 days ($10^6$ CFU $mL^{-1}$), and 1 % solution of the same was used for dipping the cuttings overnight as well as for foliar application. The cuttings after treatment were planted in polybags filled with standard potting mixture. Foliar application was done at 45, 75 and 90 DAP. The growth parameters were recorded 90 DAP.

### *Effects of selected PNPPFM isolates on drought tolerance of black pepper cuttings*

To test the drought tolerance potential of the PNPPFM isolates another set of cuttings which were given the same treatments were subjected to drought. Following the foliar spray applied 90 DAP, the plants were irrigated to field capacity for 15 days, after which irrigation was discontinued. The plantlets were observed for the onset of wilting and the physiological parameters were recorded at 60 - 65 % Relative Water Content (RWC) of leaves. cell membrane integrity (CMI), chlorophyll stability index (CSI), proline content, catalase, super oxide dismutase (SOD) and peroxidase activity of the leaves were measured.

### *Statistical analysis*

The experimental data were evaluated using ANOVA based on a one-factor completely randomized design (CRD) with the aid of GRAPES software. For treatment comparisons, the least significant differences were assessed at the 5 % significance level.

## Results

### *Isolation and characterisation of PPFMs*

The PPFM isolates with distinct colony morphology and pink pigmentation obtained from the phyllosphere and rhizosphere of black pepper plants were designated as PNPPFMs and 60 isolates were selected for further studies. All isolates displayed pink pigmentation and tested positive for catalase and oxidase. The colonies were small to medium in size, typically round, with most showing light to medium pink coloration due to their pigmentation. All isolates were Gram-negative rods.

### *Assessment of plant growth promotion traits*

IAA production was detected in 33 PNPPFM isolates from phyllosphere and 4 isolates from rhizosphere. The IAA production both in the absence and presence of tryptophan in the culture filtrate exhibited considerable variability, with levels ranging from 10.02 - 51.25 µg mL$^{-1}$ and 10.06 - 60.00 µg mL$^{-1}$ respectively (Fig. 2).

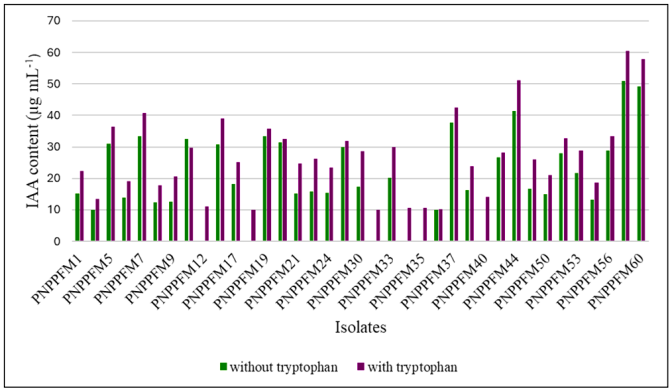


**Fig. 2.** IAA production by PNPPFM isolates from black pepper.

Further in vitro studies were done only on 30 isolates producing higher quantity of IAA. All 30 isolates produced varying amounts of gibberellic acid, ranging from 1.81 - 29.19 µg mL$^{-1}$ while only 19 isolates produced ammonia extracellularly (1.43 - 7.07 µmol mL$^{-1}$) (Table 1). The highest extracellular ammonia of 7.07 µmol mL$^{-1}$ of culture was recorded by PNPPFM 59 followed by PNPPFM 60 (6.92 µmol mL$^{-1}$). All selected isolates showed ACC deaminase activity, with values ranging from 0.89 - 13.94 µg mL$^{-1}$. Of the 30 isolates, the highest ACC deaminase activity was recorded in PNPPFM 60 (13.94 µg mL$^{-1}$) followed by PNPPFM 59 (12.64 µg mL$^{-1}$).

### *Assessment of drought tolerance potential of the selected isolates in vitro*

All the isolates were able to grow up to 30 % concentration of PEG 6000 efficiently. The absorbance of the culture broth at 600 nm in the absence of PEG 6000 ranged from 0.45 - 0.98. Absorbance at 5 % PEG 6000 was 0.45 - 0.99, at 10 % was 0.36 - 0.78, at 20 % was 0.21 - 0.60 and at 30 % PEG 6000 was 0.10 - 0.50. Absorbance at 40 % PEG 6000 concentration ranged from 0.01 - 0.45. The highest absorbance value was recorded in PNPPFM 60 at all concentrations of PEG 6000, and it could grow in the presence of 40 % PEG (0.45) indicating its drought tolerance potential (Table 2).

The ranking of PNPPFM isolates was carried out based on the production of IAA, GA, extracellular ammonia, ACC deaminase activity and drought tolerance potential. The better performing isolates PNPPFM 60, PNPPFM 59 and PNPPFM 44 were selected for molecular identification and subsequent pot culture experiment.

### *Molecular identification of the selected isolates*

The selected isolates; PNPPFM 60, PNPPFM 59 and PNPPFM 44 were identified as *Methylobacterium aerolatum*, *Methylorubrum aminovorans,* and *M. zatmanii* respectively based on 16S rRNA sequencing using universal primers. Phylogenetic tree based on the 16S rRNA gene sequences of these isolates is presented in Fig. 3.

### *Effects of selected PNPPFM isolates on plant growth promotion of black pepper cuttings*

All the biometric parameters improved due to the treatment with PNPPFMs than control. Highest number of leaves (4.66), shoot length (28.30 cm), root length (17.70 cm), number of roots, fresh weight of roots (1.01 g), dry weight of roots (0.40 g), fresh weight of newly emerged shoot and leaves (10.74 g) and dry weight of newly emerged shoot and leaves (2.96 g) were recorded in *M. aerolatum* PNPPFM 60 treated cuttings (Table 3, 4; Fig. 4, 5). The growth parameters recorded were the lowest in control plants.

### *Effects of selected PNPPFM isolates on drought tolerance of black pepper cuttings*

The symptoms of withholding irrigation were evident in the control plants after 3 days with RWC of 64.88 % while the PPFM treated plants were healthy and showed an RWC of 84.40 % on the 3$^{rd}$ day. The physiological parameters recorded on the 4$^{th}$ day after withholding the irrigation showed significantly better result in the treatment with *M. aerolatum* PNPPFM 60. The highest CMI (88.21 %) and CSI (78.26 %) recorded in *M. aerolatum* PNPPFM 60 was statistically on par with *M. aminovorans* PNPPFM 59 (86.56 % and 75.77 %) on the 4$^{th}$ day.

**Table 1.** Production of plant growth promoting substances by PNPPFM isolates

| Sl. No | Isolate code no. | Gibberellic acid* (µg mL$^{-1}$) | Ammonia production* (µmol | ACC deaminase production* (µg mL$^{-1}$) |
|---|---|---|---|---|
| 1 | PNPPFM 1 | 3.72 ± 0.09$^{x}$ | 2.04 ± 0.01$^{k}$ | 6.67 ± 0.49$^{g}$ |
| 2 | PNPPFM 5 | 12.08 ± 0.02$^{f}$ | 1.74 ± 0.00$^{m}$ | 5.00 ± 0.20$^{hij}$ |
| 3 | PNPPFM 6 | 3.42 ± 0.02$^{y}$ | ND | 11.15 ± 0.01$^{c}$ |
| 4 | PNPPFM 7 | 13.04 ± 0.10$^{d}$ | ND | 8.49 ± 0.00$^{e}$ |
| 5 | PNPPFM 8 | 2.40 ± 0.02$^{A}$ | ND | 7.64 ± 0.00$^{f}$ |
| 6 | PNPPFM 9 | 1.81 ± 0.02$^{B}$ | ND | 4.28 ± 0.00$^{jk}$ |
| 7 | PNPPFM 11 | 10.67 ± 0.12$^{i}$ | 1.89 ± 0.01$^{l}$ | 4.84 ± 0.01$^{ijk}$ |
| 8 | PNPPFM 14 | 12.39 ± 0.04$^{e}$ | 4.07 ± 0.00$^{f}$ | 5.67 ± 0.19$^{h}$ |
| 9 | PNPPFM 17 | 6.68 ± 0.15$^{p}$ | ND | 8.39 ± 0.04$^{e}$ |
| 10 | PNPPFM 19 | 8.60 ± 0.03$^{l}$ | ND | 12.89 ± 0.51$^{b}$ |
| 11 | PNPPFM 20 | 8.81 ± 0.00$^{k}$ | 5.49 ± 0.00$^{d}$ | 1.10 ± 0.00$^{m}$ |
| 12 | PNPPFM 21 | 4.36 ± 0.03$^{v}$ | 1.44 ± 0.00$^{o}$ | 0.89 ± 0.00$^{m}$ |
| 13 | PNPPFM 22 | 4.67 ± 0.04$^{t}$ | ND | 9.85 ± 0.20$^{d}$ |
| 14 | PNPPFM 24 | 4.50 ± 0.04$^{u}$ | 1.43 ± 0.00$^{o}$ | 12.87 ± 0.00$^{b}$ |
| 15 | PNPPFM 26 | 9.79 ± 0.02$^{j}$ | 2.19 ± 0.00$^{j}$ | 4.84 ± 0.01$^{ijk}$ |
| 16 | PNPPFM 29 | 13.52 ± 0.02$^{c}$ | ND | 5.48 ± 0.01$^{hi}$ |
| 17 | PNPPFM 30 | 6.55 ± 0.06$^{q}$ | 2.45 ± 0.00$^{h}$ | 12.56 ± 1.66$^{b}$ |
| 18 | PNPPFM 33 | 7.44 ± 0.03$^{o}$ | 1.51 ± 0.01$^{n}$ | 8.53 ± 0.12$^{e}$ |
| 19 | PNPPFM 37 | 14.94 ± 0.02$^{b}$ | 4.13 ± 0.01$^{e}$ | 4.57 ± 0.39$^{jk}$ |
| 20 | PNPPFM 39 | 5.09 ± 0.01$^{s}$ | ND | 1.20 ± 0.16$^{m}$ |
| 21 | PNPPFM 41 | 7.75 ± 0.03$^{n}$ | 5.54 ± 0.00$^{c}$ | 4.27 ± 0.02$^{k}$ |
| 22 | PNPPFM 44 | 12.29 ± 0.01$^{e}$ | 3.76 ± 0.01$^{g}$ | 11.11 ± 0.42$^{c}$ |
| 23 | PNPPFM 47 | 5.76 ± 0.02$^{r}$ | 1.74 ± 0.00$^{m}$ | 11.16 ± 0.03$^{c}$ |
| 24 | PNPPFM 50 | 3.97 ± 0.08$^{w}$ | 1.39 ± 0.01$^{p}$ | 2.38 ± 0.01$^{l}$ |
| 25 | PNPPFM 52 | 11.32 ± 0.02$^{h}$ | 2.40 ± 0.00$^{i}$ | 2.37 ± 0.00$^{l}$ |
| 26 | PNPPFM 53 | 7.75 ± 0.03$^{n}$ | ND | 11.07 ± 0.16$^{c}$ |
| 27 | PNPPFM 54 | 2.59 ± 0.02$^{z}$ | ND | 1.32 ± 0.04$^{m}$ |
| 28 | PNPPFM 56 | 11.58 ± 0.06$^{g}$ | 1.74 ± 0.00$^{m}$ | 9.27 ± 0.01$^{d}$ |
| 29 | PNPPFM 59 | 7.93 ± 0.02$^{m}$ | 7.07 ± 0.01$^{a}$ | 12.64 ± 0.05$^{b}$ |
| 30 | PNPPFM 60 | 29.19 ± 0.03$^{a}$ | 6.92 ± 0.00$^{b}$ | 13.94 ± 0.01$^{a}$ |
| | CD | 0.115 | 0.013 | 0.729 |
| | SE(d) ± | 0.056 | 0.006 | 0.357 |

Different letters in superscripts in a column indicate significantly different values, *****Mean of 3 independent replications and **ND**- Not detected.

**Table 2.** Growth of PNPPFM isolates from black pepper under PEG 6000 induced moisture stress.

| Sl. No | Isolate | $OD_{600}$ at PEG 0 %* | $OD_{600}$ at PEG 5 %* | $OD_{600}$ at PEG 10 %* | $OD_{600}$ at PEG 20 %* | $OD_{600}$ at PEG 30 %* | $OD_{600}$ at PEG 40 %* |
|---|---|---|---|---|---|---|---|
| 1 | PNPPFM1 | 0.81 ± 0.003$^{i}$ | 0.72 ± 0.01$^{j}$ | 0.69 ± 0.01$^{de}$ | 0.41 ± 0.01$^{hi}$ | 0.33 ± 0.02$^{de}$ | 0.04 ± 0.001$^{q}$ |
| 2 | PNPPFM5 | 0.81 ± 0.001$^{i}$ | 0.75 ± 0.01$^{h}$ | 0.61 ± 0.01$^{g}$ | 0.32 ± 0.01$^{k}$ | 0.22 ± 0.02$^{Hi}$ | 0.06 ± 0.001$^{p}$ |
| 3 | PNPPFM6 | 0.81 ± 0.007$^{i}$ | 0.81 ± 0.01$^{f}$ | 0.69 ± 0.01$^{de}$ | 0.45 ± 0.01$^{g}$ | 0.36 ± 0.02$^{bc}$ | 0.11 ± 0.001$^{l}$ |
| 4 | PNPPFM7 | 0.76 ± 0.001$^{mn}$ | 0.75 ± 0.00$^{hi}$ | 0.65 ± 0.01$^{f}$ | 0.33 ± 0.01$^{k}$ | 0.21 ± 0.02$^{I}$ | 0.06 ± 0.001$^{p}$ |
| 5 | PNPPFM8 | 0.79 ± 0.001$^{j}$ | 0.78 ± 0.01$^{g}$ | 0.54 ± 0.01$^{i}$ | 0.35 ± 0.01$^{j}$ | 0.15 ± 0.02$^{J}$ | 0.08 ± 0.001$^{o}$ |
| 6 | PNPPFM9 | 0.54 ± 0.001$^{t}$ | 0.54 ± 0.01$^{o}$ | 0.41 ± 0.01$^{m}$ | 0.25 ± 0.01$^{m}$ | 0.10 ± 0.02$^{L}$ | 0.01 ± 0.001$^{r}$ |
| 7 | PNPPFM11 | 0.76 ± 0.001$^{lm}$ | 0.75 ± 0.01$^{hi}$ | 0.52 ± 0.01$^{j}$ | 0.39 ± 0.01$^{i}$ | 0.21 ± 0.02$^{I}$ | 0.01 ± 0.001$^{n}$ |
| 8 | PNPPFM14 | 0.88 ± 0.00$^{e}$ | 0.86 ± 0.00$^{d}$ | 0.66 ± 0.01$^{f}$ | 0.51 ± 0.01$^{d}$ | 0.35 ± 0.02$^{cd}$ | 0.35 ± 0.001$^{e}$ |
| 9 | PNPPFM17 | 0.85 ± 0.001$^{g}$ | 0.84 ± 0.00$^{e}$ | 0.65 ± 0.01$^{f}$ | 0.49 ± 0.01$^{ef}$ | 0.32 ± 0.02$^{Ef}$ | 0.25 ± 0.001$^{g}$ |
| 10 | PNPPFM19 | 0.90 ± 0.007$^{c}$ | 0.90 ± 0.00$^{b}$ | 0.78 ± 0.01$^{a}$ | 0.59 ± 0.01$^{ab}$ | 0.39 ± 0.02$^{b}$ | 0.30 ± 0.001$^{f}$ |
| 11 | PNPPFM20 | 0.76 ± 0.001$^{m}$ | 0.75 ± 0.00$^{hi}$ | 0.65 ± 0.01$^{f}$ | 0.49 ± 0.01$^{ef}$ | 0.25 ± 0.02$^{G}$ | 0.09 ± 0.001$^{o}$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 12 | PNPPFM21 | 0.46 ± 0.00$^{v}$ | 0.45 ± 0.01$^{q}$ | 0.39 ± 0.01$^{n}$ | 0.26 ± 0.01$^{m}$ | 0.10 ± 0.02$^{L}$ | 0.01 ± 0.001$^{r}$ |
| 13 | PNPPFM22 | 0.61 ± 0.001$^{r}$ | 0.59 ± 0.01$^{n}$ | 0.36 ± 0.01$^{o}$ | 0.21 ± 0.01$^{n}$ | 0.14 ± 0.02$^{Jk}$ | 0.05 ± 0.001$^{pq}$ |
| 14 | PNPPFM24 | 0.75 ± 0.00$^{n}$ | 0.74 ± 0.01$^{ij}$ | 0.61 ± 0.01$^{g}$ | 0.48 ± 0.01$^{f}$ | 0.29 ± 0.02$^{F}$ | 0.01 ± 0.001$^{n}$ |
| 15 | PNPPFM26 | 0.76 ± 0.001$^{l}$ | 0.75 ± 0.01$^{h}$ | 0.58 ± 0.01$^{h}$ | 0.39 ± 0.01$^{i}$ | 0.25 ± 0.02$^{G}$ | 0.01 ± 0.001$^{m}$ |
| 16 | PNPPFM29 | 0.86 ± 0.001$^{f}$ | 0.85 ± 0.01$^{de}$ | 0.68 ± 0.01$^{e}$ | 0.51 ± 0.01$^{de}$ | 0.36 ± 0.02$^{bc}$ | 0.20 ± 0.001$^{h}$ |
| 17 | PNPPFM30 | 0.78 ± 0.001$^{k}$ | 0.75 ± 0.01$^{h}$ | 0.54 ± 0.01$^{i}$ | 0.32 ± 0.01$^{k}$ | 0.15 ± 0.02$^{J}$ | 0.04 ± 0.001$^{q}$ |
| 18 | PNPPFM33 | 0.45 ± 0.001$^{w}$ | 0.45 ± 0.01$^{q}$ | 0.36 ± 0.01$^{o}$ | 0.29 ± 0.01$^{l}$ | 0.11 ± 0.02$^{Kl}$ | 0.01 ± 0.001$^{r}$ |
| 19 | PNPPFM37 | 0.59 ± 0.00$^{s}$ | 0.59 ± 0.01$^{n}$ | 0.40 ± 0.01$^{mn}$ | 0.30 ± 0.01$^{l}$ | 0.14 ± 0.02$^{Jk}$ | 0.08 ± 0.001$^{o}$ |
| 20 | PNPPFM39 | 0.89 ± 0.001$^{d}$ | 0.88 ± 0.01$^{c}$ | 0.69 ± 0.01$^{de}$ | 0.55 ± 0.01$^{c}$ | 0.30 ± 0.02$^{Ef}$ | 0.39 ± 0.001$^{d}$ |
| 21 | PNPPFM41 | 0.86 ± 0.002$^{f}$ | 0.85 ± 0.01$^{de}$ | 0.68 ± 0.01$^{e}$ | 0.50 ± 0.01$^{def}$ | 0.39 ± 0.02$^{b}$ | 0.17 ± 0.001$^{i}$ |
| 22 | PNPPFM44 | 0.90 ± 0.00$^{cd}$ | 0.89 ± 0.01$^{bc}$ | 0.72 ± 0.01$^{c}$ | 0.59 ± 0.01$^{ab}$ | 0.50 ± 0.02$^{a}$ | 0.41 ± 0.001$^{c}$ |
| 23 | PNPPFM47 | 0.64 ± 0.007$^{p}$ | 0.66 ± 0.01$^{l}$ | 0.50 ± 0.01$^{k}$ | 0.48 ± 0.01$^{f}$ | 0.21 ± 0.02$^{I}$ | 0.09 ± 0.001$^{o}$ |
| 24 | PNPPFM50 | 0.68 ± 0.00$^{o}$ | 0.69 ± 0.01$^{k}$ | 0.54 ± 0.01$^{i}$ | 0.49 ± 0.01$^{def}$ | 0.31 ± 0.02$^{Ef}$ | 0.01 ± 0.001$^{m}$ |
| 25 | PNPPFM52 | 0.64 ± 0.007$^{q}$ | 0.64 ± 0.01$^{m}$ | 0.51 ± 0.01$^{jk}$ | 0.42 ± 0.01$^{h}$ | 0.32 ± 0.02$^{Ef}$ | 0.01 ± 0.001$^{n}$ |
| 26 | PNPPFM53 | 0.52 ± 0.00$^{u}$ | 0.52 ± 0.01$^{p}$ | 0.48 ± 0.01$^{l}$ | 0.36 ± 0.01$^{j}$ | 0.25 ± 0.02$^{Gh}$ | 0.01 ± 0.001$^{r}$ |
| 27 | PNPPFM54 | 0.82 ± 0.002$^{h}$ | 0.82 ± 0.01$^{f}$ | 0.66 ± 0.01$^{f}$ | 0.58 ± 0.01$^{b}$ | 0.33 ± 0.02$^{De}$ | 0.14 ± 0.001$^{k}$ |
| 28 | PNPPFM56 | 0.85 ± 0.00$^{g}$ | 0.82 ± 0.01$^{f}$ | 0.71 ± 0.01$^{d}$ | 0.58 ± 0.01$^{b}$ | 0.33 ± 0.02$^{De}$ | 0.15 ± 0.001$^{j}$ |
| 29 | PNPPFM59 | 0.91 ± 0.002$^{b}$ | 0.90 ± 0.01$^{b}$ | 0.75 ± 0.01$^{b}$ | 0.60 ± 0.01$^{a}$ | 0.50 ± 0.02$^{a}$ | 0.42 ± 0.001$^{b}$ |
| 30 | PNPPFM60 | 0.98 ± 0.002$^{a}$ | 0.99 ± 0.01$^{a}$ | 0.76 ± 0.01$^{b}$ | 0.59 ± 0.01$^{ab}$ | 0.50 ± 0.02$^{a}$ | 0.45 ± 0.001$^{a}$ |
| | **CD** | 0.006 | 0.015 | 0.016 | 0.016 | 0.033 | 0.002 |
| | **SE(d) ±** | 0.003 | 0.005 | 0.006 | 0.006 | 0.012 | 0.001 |

Different letters in superscripts in a column indicate significantly different values *Mean of 3 independent replications.

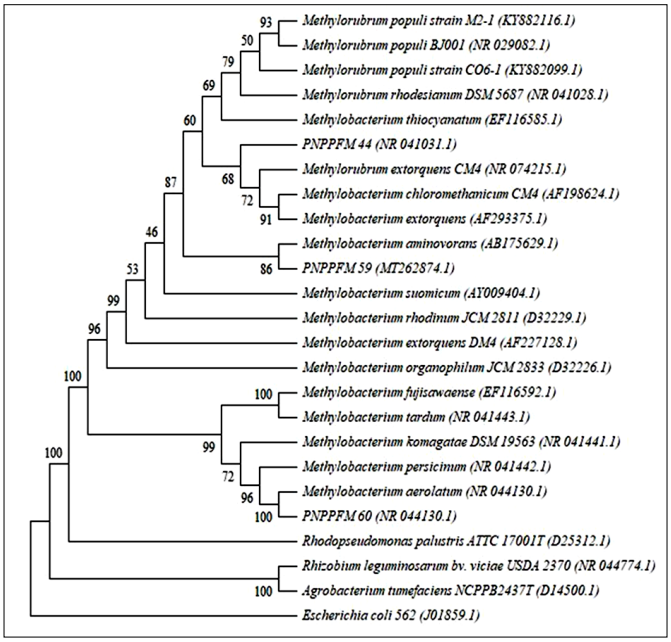


**Fig. 3.** Phylogenetic relatedness based on the 16S rRNA gene sequences of PNPPFM isolates and other recognized species of *Methylobacterium* using neighbor-joining method. The data of type strains of *Methylobacterium* species and other genera were from GenBank database (the accession numbers are given in parentheses).

**Table 3.** Biometric parameters of black pepper cuttings treated with PNPPFM isolates

| Treatments | No. of leaves* | Shoot length* (cm) | Root length* (cm) |
|---|---|---|---|
| PNPPFM 60 | 4.66 ± 0.57$^{a}$ | 28.30 ± 2.42$^{a}$ | 17.70 ± 4.81$^{a}$ |
| PNPPFM 59 | 3.66 ± 1.15$^{ab}$ | 23.20 ± 3.70$^{bc}$ | 11.10 ± 4.55$^{b}$ |
| PNPPFM 44 | 3.33 ± 0.57$^{b}$ | 26.66 ± 3.05$^{ab}$ | 11.56 ± 3.25$^{b}$ |
| PPFM 38 (Ref.) | 2.66 ± 0.57$^{bc}$ | 21.26 ± 1.65$^{cd}$ | 8.96 ± 0.23$^{b}$ |
| Sterile water | 1.33 ± 0.57$^{d}$ | 17.60 ± 1.97$^{de}$ | 2.70 ± 1.30$^{c}$ |
| AMS media | 1.66 ± 0.57$^{cd}$ | 15.43 ± 1.25$^{e}$ | 2.46 ± 0.70$^{c}$ |
| Absolute control | 1.66 ± 0.57$^{cd}$ | 19.70 ± 3.03$^{cde}$ | 2.76 ± 1.17$^{c}$ |
| CD | 1.20 | 4.50 | 5.04 |
| SE(d) ± | 0.56 | 2.09 | 2.35 |

Different letters in superscripts in a column indicate significantly different values, *****Mean of 3 independent replications.

The highest proline content (78.67 µg $g^{-1}$ tissue) and drought related enzymes like SOD (0.45 activity $g^{-1}$ $min^{-1}$), catalase (18.48 µg $H_2O_2$ $g^{-1}$ $min^{-1}$) and peroxidase activity (47.18 $g^{-1}min^{-1}$) was recorded in the plants treated with *M. aerolatum* PNPPFM 60 while the control plants recorded the least (Table 5, 6).

**Table 4.** Effect of PNPPFM isolates on rooting and shooting of black pepper cuttings

| Treatments | No. of primary roots* | No. of secondary roots* | Fresh wt of roots (g) * | Dry wt of roots (g) * | Fresh wt of shoot (g) * | Dry wt of shoot (g) * |
|---|---|---|---|---|---|---|
| PNPPFM 60 | 14.66 ± 3.05$^{a}$ | 138.33 ± 34.59$^{a}$ | 1.01 ± 0.22$^{a}$ | 0.40 ± 0.21$^{a}$ | 10.74 ± 2.29$^{a}$ | 2.96 ± 0.80 |
| PNPPFM 59 | 10.00 ± 3.60$^{b}$ | 123 ± 27.83$^{a}$ | 1.00 ± 0.35$^{ab}$ | 0.28 ± 0.15$^{ab}$ | 5.71 ± 1.706$^{bc}$ | 1.24 ± 0.60 |
| PNPPFM 44 | 7.00 ± 2.00$^{b}$ | 62.00 ± 20.88$^{b}$ | 0.40 ± 0.12$^{c}$ | 0.10 ± 0.01$^{bc}$ | 7.36 ± 2.24$^{abc}$ | 1.60 ± 0.44 |
| PPFM 38 (Ref.) | 7.33 ± 1.52$^{b}$ | 62.66 ± 22.54$^{b}$ | 0.69 ± 0.19$^{bc}$ | 0.12 ± 0.16$^{bc}$ | 8.94 ± 1.90$^{ab}$ | 2.89 ± 1.47 |
| Sterile water | 2.33 ± 0.57$^{c}$ | 3.66 ± 2.51$^{c}$ | 0.02 ± 0.00$^{d}$ | 0.01 ± 0.00$^{c}$ | 4.20 ± 0.93$^{c}$ | 0.89 ± 0.36 |

| | | | | | | |
|---|---|---|---|---|---|---|
| AMS media | 2.00 ± 0.00$^{c}$ | 3.00 ± 2.64$^{c}$ | 0.02 ± 0.01$^{d}$ | 0.01 ± 0.00$^{c}$ | 6.42 ± 0.86$^{bc}$ | 1.40 ± 0.51 |
| Absolute control | 2.66 ± 0.57$^{c}$ | 5.66 ± 4.04$^{c}$ | 0.04 ± 0.02$^{d}$ | 0.02 ± 0.01$^{c}$ | 6.90 ± 3.11$^{bc}$ | 2.06 ± 1.68 |
| CD | 3.58 | 35.90 | 0.31 | 0.20 | 3.51 | NS |
| SE(d) ± | 1.67 | 16.74 | 0.14 | 0.09 | 1.63 | 0.79 |

Different letters in superscripts in a column indicate significantly different values, *****Mean of 3 independent replications, **NS**- nonsignificant.


**Fig. 4.** Growth of black pepper cuttings on inoculation with PNPPFMs (90 DAP). **T1**- PNPPFM 60, **T2**- PNPPFM 59, **T3**- PNPPFM 44, **T4**- PPFM 38 (Ref. culture), **T5**- Sterile water, **T6**- Uninoculated media, **T7**- Absolute control.

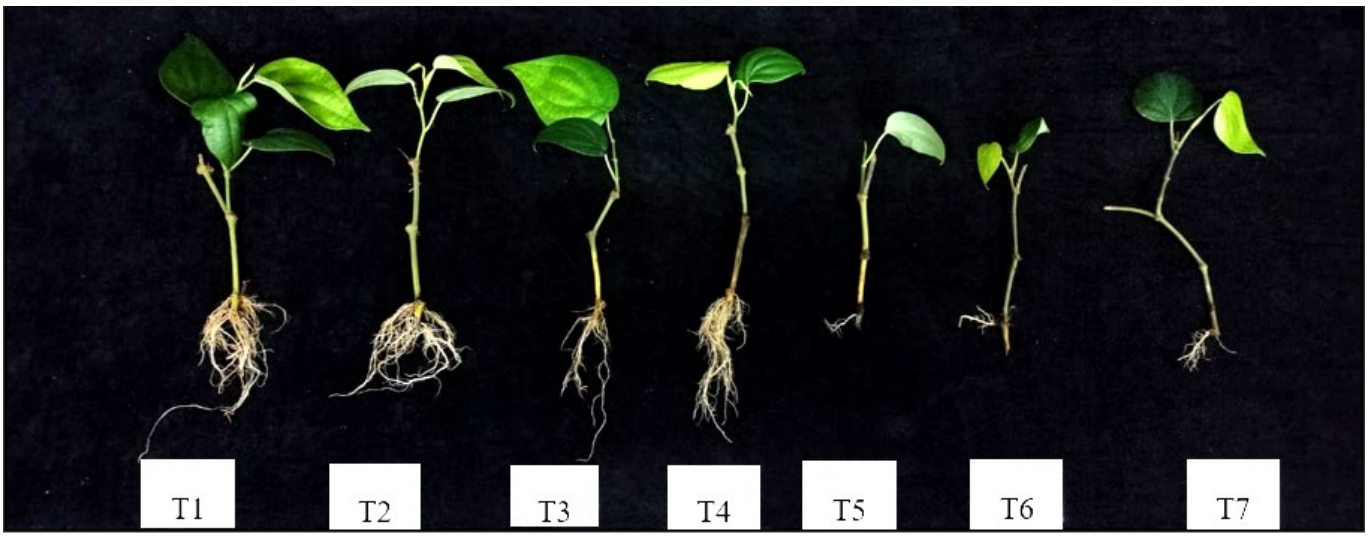


**Fig. 5.** Effect of PNPPFM application on rooting of pepper cuttings (90 DAP). **T1**- PNPPFM 60, **T2**- PNPPFM 59, **T3**- PNPPFM 44, **T4**- PPFM 38 (Ref. culture), **T5**- Sterile water, **T6**- Uninoculated media and **T7**- Absolute control.

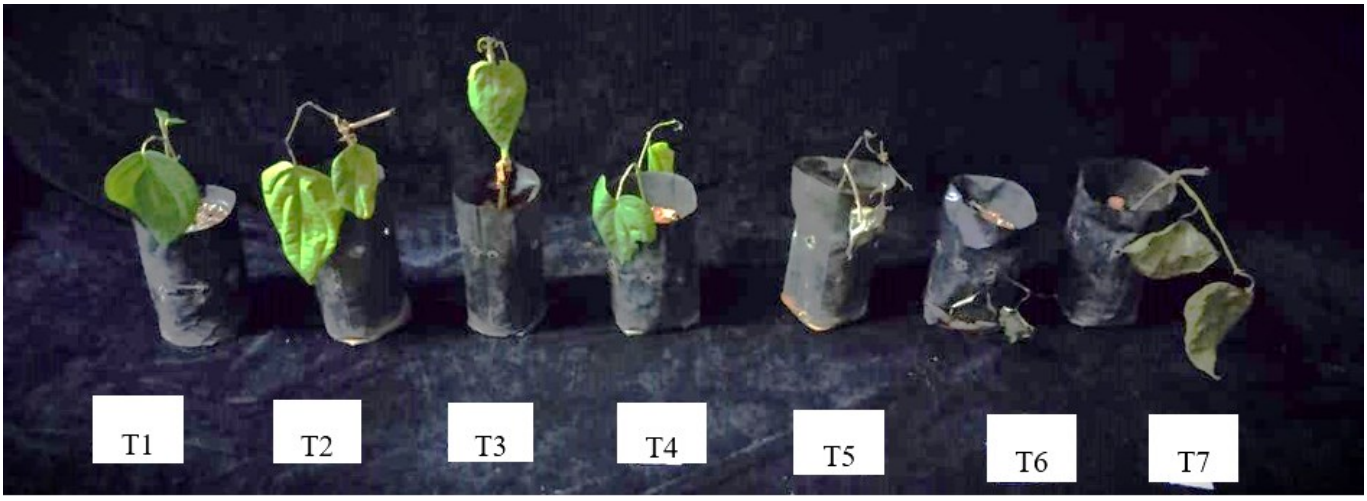


**Fig. 6.** Drought tolerance in black pepper mediated by PNPPFMs after 7 days of withholding irrigation. **T1**- PNPPFM 60, **T2**- PNPPFM 59, **T3**- PNPPFM 44, **T4** - PPFM 38 (Ref. culture), **T5**- Sterile water, **T6**- Uninoculated media and **T7**- Absolute control.

**Table 5.** Effect of PNPPFM isolates on physiological parameters of black pepper plantlets under drought stress

| Treatments | RWC on 7th day* (%) | RWC on 14th day* (%) | CMI on 7th day* (%) | CMI on 14th day* (%) | Chlorophyll stability index (%) |
|---|---|---|---|---|---|
| PNPPFM 60 | 84.40 ± 0.73$^{a}$ | 78.50 ± 0.91$^{a}$ | 88.21 ± 2.58$^{a}$ | 79.34 ± 1.99$^{a}$ | 78.26 ± 1.08$^{a}$ |
| PNPPFM 59 | 82.07 ± 1.28$^{b}$ | 73.17 ± 0.28$^{b}$ | 86.56 ± 0.75$^{a}$ | 78.01 ± 1.80$^{a}$ | 75.77 ± 0.99$^{b}$ |
| PNPPFM 44 | 80.27 ± 0.47$^{b}$ | 70.02 ± 3.08$^{b}$ | 80.74 ± 2.29$^{b}$ | 73.72 ± 2.20$^{b}$ | 72.79 ± 0.66$^{c}$ |
| PPFM 38 (Ref.) | 82.43 ± 1.35$^{ab}$ | 72.98 ± 0.73$^{b}$ | 86.42 ± 2.06$^{a}$ | 78.13 ± 2.00$^{a}$ | 72.46 ± 1.24$^{c}$ |
| Sterile water | 68.48 ± 2.04$^{c}$ | 56.82 ± 4.12$^{c}$ | 69.33 ± 3.03$^{c}$ | 35.78 ± 1.17$^{c}$ | 51.70 ± 0.72$^{d}$ |
| AMS media | 67.91 ± 1.13$^{c}$ | 57.68 ± 2.10$^{c}$ | 66.64 ± 1.54$^{cd}$ | 32.40 ± 1.60$^{d}$ | 49.56 ± 0.38$^{e}$ |
| Absolute control | 64.88 ± 1.15$^{d}$ | 55.52 ± 1.65$^{c}$ | 63.10 ± 1.48$^{d}$ | 30.21 ± 2.10$^{d}$ | 49.89 ± 0.49$^{e}$ |
| CD | 2.19 | 3.92 | 3.66 | 3.27 | 1.49 |
| SE(d) ± | 1.02 | 1.83 | 1.70 | 1.52 | 0.69 |

Different letters in superscripts in a column indicate significantly different values, *****Mean of 3 independent replications.

**Table 6.** Effect of PNPPFM isolates on enzyme activity of black pepper plantlets under drought stress

| Treatments | Catalase activity* (µg $H_2O_2$ $g^{-1}$ $min^{-1}$) | Proline* (µg g tissue$^{-1}$) | SOD* (activity $g^{-1}$ $min^{-1}$) | POD* (activity $g^{-1}$ $min^{-1}$) |
|---|---|---|---|---|
| PNPPFM 60 | 18.48 ± 0.21$^{a}$ | 78.67 ± 0.05$^{a}$ | 0.45 ± 0.00$^{a}$ | 47.18 ± 0.78$^{a}$ |
| PNPPFM 59 | 17.99 ± 0.24$^{b}$ | 71.32 ± 2.05$^{b}$ | 0.42 ± 0.00$^{b}$ | 39.14 ± 0.12$^{b}$ |
| PNPPFM 44 | 15.44 ± 0.12$^{c}$ | 63.85 ± 0.81$^{c}$ | 0.39 ± 0.01$^{c}$ | 26.95 ± 0.41$^{d}$ |
| PPFM 38 (Ref.) | 17.92 ± 0.12$^{b}$ | 72.97 ± 0.99$^{b}$ | 0.41 ± 0.00$^{c}$ | 36.81 ± 0.00$^{c}$ |
| Sterile water | 7.29 ± 0.12$^{d}$ | 32.03 ± 0.09$^{d}$ | 0.20 ± 0.01$^{e}$ | 15.04 ± 0.04$^{e}$ |
| AMS media | 6.87 ± 0.12$^{e}$ | 30.64 ± 1.93$^{d}$ | 0.23 ± 0.00$^{d}$ | 13.08 ± 0.72$^{f}$ |
| Absolute control | 6.65 ± 0.12$^{e}$ | 23.98 ± 0.10$^{e}$ | 0.19 ± 0.00$^{e}$ | 13.08 ± 0.53$^{f}$ |
| CD | 0.28 | 2.05 | 0.015 | 0.82 |
| SE(d) ± | 0.13 | 0.96 | 0.007 | 0.38 |

Different letters in superscripts in a column indicate significantly different values, *****Mean of 3 independent replications.

## Discussions

The positive impact of PPFMs on the plant growth and yield is demonstrated by several workers. In our study 60 distinct PNPPFM isolates obtained from black pepper were characterised for oxidase and catalase. Similar results were documented in paddy where five PPFM isolates were positive for oxidase, urease, catalase and indole production (20). These biochemical traits exhibited by the isolates might facilitate the flourishing of PPFM bacteria in the aerial parts of plants (2).

Many plants associated bacteria produce plant hormones such as IAA, GA and cytokinins through plant-secreted precursors which help in cell elongation and better plant growth. In our investigation *M. aerolatum* PNPPFM 60 produced the highest GA (29.19 µg $mL^{-1}$) and IAA (60.00 µg $mL^{-1}$). Gibberellins play a crucial role in germination of seeds, development of shoot and leaves, floral induction etc. GA and other plant hormones are directly effective in enhancing the elongation of shoots in plants. GA production in *Methylobacterium* in the range of 10.9 µg $mL^{-1}$ - 106.97 µg $mL^{-1}$ was reported from vegetable crops (21). IAA concentrations of 2.33 and 4.03 µg $mL^{-1}$ in the absence and presence of L-tryptophan, respectively was reported from *Methylobacterium* isolates of rice (22). Auxin is a plant growth promoting root hormone which enhance crop growth and productivity by promoting root development. It can increase the biotic and abiotic stress tolerance potential of the plants. Various Auxin Response Factors (ARFs) are essential for regulating soluble sugar content, enhancing root development and sustaining chlorophyll levels during drought and saline stress, helping plants adapt to these adverse conditions. Numerous reports are available on the production of IAA by PPFMs in varying concentrations *in vitro* (20, 23, 24). The production of IAA by *M. aerolatum* PNPPFM 60 and the increased rooting may have improved the drought tolerance potential of PNPPFM 60 treated pepper cuttings in this experiment.

The bacteria on the leaves produce extracellular ammonia the accumulation of which supplies nitrogen to their hosts thereby improving the shoot, root length and plant biomass. Extracellular ammonia production was recorded by 19 PNPPFM isolates in the present study (1.43 - 7.07 µmol $mL^{-1}$).

Environmental stresses trigger the production of the stress hormone, ethylene (Et) in plants as part of its defence mechanism, helping the plant adapt or respond to the unfavourable conditions. In abiotic stress, like drought or high salinity, Et can mediate processes like leaf senescence, root growth modulation and stomatal closure, which can help conserve water and energy. Under biotic stress, such as pathogen invasion, Et signalling works in coordination with other hormones like salicylic acid (SA) and jasmonic acid (JA) to mount a defense response. However, excessive Et production can sometimes exacerbate the stress by promoting premature aging or excessive tissue damage (25). To regulate this, plants use enzymes like ACC deaminase, which lower Et levels by degrading its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). This helps the plant strike a balance between stress response and growth. The enzymatic action of ACC deaminase leads

to the creation of α-ketobutyrate and ammonia. This process reduces ACC concentrations and prevent the overproduction of ethylene under various stress conditions. This mechanism is highly effective in promoting plant tolerance to stress conditions. PPFMs have been found to function as a reservoir for ACC deaminase enzyme mitigating the negative impacts of diverse stresses (26). In our study all selected PNPPFM isolates showed ACC deaminase activity in the range of 0.89 - 13.94 µg $mL^{-1}$. Reports on the occurrence of ACC deaminase activity of PPFMs are made by several researchers. *M. fujisawaense* significantly reduced ethylene levels in canola seedlings, while *Methylobacterium oryzae* sp*. nov.,* an aerobic PPFM-producing bacterium found in rice stem tissues, exhibited ACC deaminase activity (27, 28).

In the moisture stress tolerance experiment all the isolates tolerated upto 30 % concentration of PEG 6000 efficiently. In a similar study with PPFMs from rice, growth of 19 PPFM isolates at 5 %, 10 % and 15 % PEG concentration and six isolates at 20 % PEG concentration was reported (24). PPFM isolates from rice recorded drought stress tolerance when screened on AMS liquid media, incorporating PEG 6000 at specific concentrations (29). Growth at 0.73 MPa was used as the criterion to select *Methylobacterium* strains from lentil tolerant to water deficit stress (30).

Best performing isolates were selected by determining the weighted average of plant growth hormones and extracellular ammonia, ACC deaminase activity and growth on PEG 6000 under *in vitro* conditions. The isolates PNPPFM 60, PNPPFM 59 and PNPPFM 44 demonstrated high efficiency and were subsequently identified using 16S rRNA sequencing. 16S rRNA gene sequence analysis suggest that the isolates belong to the genera *Methylobacterium* and *Methylorubrum*. Phylogenetic study of the PNPPFMs 16S rRNA sequence revealed that the highest sequence similarity of PNPPFM 60 was with *Methylobacterium aerolatum* strain 5413S, PNPPFM 59 *M. aminovorans* strain NG22 and PNPPFM 44 *Methylorubrum zatmanii* strain DSM 5688. Our results imply that the phylogenetic relationships derived from 16S rRNA sequencing correspond with conventional taxonomic classification methods.

The pot culture experiment showed that the application of PNPPFM isolates by cutting dip and foliar spray significantly improved the growth and establishment of the cuttings. The study revealed that PNPPFM isolates had a notable positive effect on both biometric and physiological parameters, especially when stress was induced by withholding irrigation. It has been reported that the application of PPFMs in Kerala conditions led to increased water stress tolerance and improved yield in paddy (31). The use of both rhizospheric and non-rhizospheric methylotrophs as bioinoculants for various crops is well documented (32). In soybean, the application of PPFMs resulted in significantly greater plant height, leaf number, and dry weight of shoots and roots compared to the uninoculated control (33). The increase in leaf area, crop growth rate, and other growth parameters may be attributed to the hormonal activity facilitated by PPFMs. The production of plant growth hormones like IAA by PPFMs increases the proliferation of roots thus aiding plants in the accumulation of water from their surroundings, thereby enhancing their response to drought stress.

Our investigation to study the effect of inducing drought by withdrawing irrigation 15 days after a foliar spray with PNPPFM isolates showed that the treated plants (wilted on 7$^{th}$ day) were more tolerant to moisture stress than the control plants (wilted in 3 days). The foliar spray with PNPPFM bacteria effectively helped the cuttings in maintaining their RWC during drought periods. PPFM application notably improved plant cell membrane integrity under water stress conditions (34). Similarly, *Pseudomonas* spp. inoculation in maize helped maintain RWC during drought (35). Antioxidant enzyme accumulation plays a crucial role in preserving membrane stability, while extended drought stress leads to a decline in the CSI. In this study, the highest CSI of 78.26 % was recorded on cuttings treated with PNPPFM 60 while the control plants had only 49 % CSI on the 4$^{th}$ day after withholding irrigation.

During intense drought stress, proline accumulates in plants and plays a vital role as an osmolyte. Proline not only acts as an osmo-protectant for osmotic regulation but also plays a crucial role in stabilizing sub-cellular structures. It neutralises the free radicals and balances the cellular redox potential (36). In the current study, plantlets given PPFM foliar spray exhibited higher proline content (78.67 µg $g^{-1}$ tissue) compared to control (23.98 µg $g^{-1}$ tissue). The increased proline levels can enhance cytoplasmic osmotic pressure, promoting greater water uptake into different plant organs and tissues (37).

Our investigation showed that all the enzymes associated with drought tolerance were elevated in the treatments with PNPPFMs, whereas the control plants exhibited minimal enzyme activity. The highest mean SOD (0.45 activity $g^{-1}$ min$^{-1}$), catalase activity (18.48 µg $H_2O_2$ $g^{-1}$ $min^{-1}$) and peroxidase of activity (47.18 $g^{-1}$ $min^{-1}$) was recorded with PNPPFM 60 on the 4$^{th}$ day after withholding irrigation. In a similar study with snap beans, the inoculation of PPFM boosted antioxidant enzyme activity, notably increasing catalase and superoxide dismutase (SOD) levels (38).

Increased peroxidase activity is commonly observed in plants under stress, as it helps maintain cellular redox balance and reduce the buildup of harmful peroxides and free radicals. This enzyme also participates in strengthening cell walls through lignification, further enhancing the plant's ability to withstand environmental stresses (39). The regulation of peroxidase activity is therefore essential for plant survival and adaptation under adverse conditions. The elevated levels of these enzymes in treated plants indicate that PPFMs may boost the drought tolerance potential of the plants, as demonstrated by the delayed onset of drought symptoms (40). In this study the RWC of untreated plants dropped below 60 % on the third day after withholding irrigation, while the treated plants sustained their RWC, cell membrane integrity (CMI), chlorophyll stability index (CSI) and other metrics for up to 14 days without irrigation.

## Conclusion

This study establishes the growth-promoting and drought tolerance capabilities of PPFM isolates in black pepper cuttings. The findings indicate that *Methylobacterium aerolatum* PNPPFM 60, applied through a combination of a 1 % solution for cutting dips and a 1 % foliar spray of PPFM broth culture at 45, 75 and 90 days after planting (DAP), significantly enhances both growth and drought tolerance in black pepper. Further research in field conditions is needed to fully assess their potential. Nonetheless, these results suggest that PPFMs are promising bioinoculants for enhancing growth and mitigating drought stress in crop plants.

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## Authors' contributions

SAR designed the experiment, conducted statistical analysis and co-wrote the paper. YMB carried out the experimental works, conducted data analysis and wrote the paper. NC participated in its design and edited the manuscript. VIS participated in the design and coordination, carried out corrections and edited the manuscript. RB participated in the design of the study and helped in enzyme analysis and data correction. KNA gave guidance in the design of experimental work and corrected the manuscript. All authors have read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest**: Authors do not have any conflict of interests to declare.

**Ethical issues**: None

## AI Declaration

During the preparation of this work the author(s) used ChatGPT AI tool to improve language and check grammar. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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