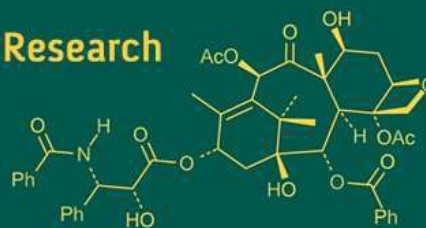


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## Isolation and identification of Pink-Pigmented Facultative Methylophs (PPFMs) and evaluation of their potential as biocontrol agents against *Colletotrichum lindemuthianum* causing anthracnose of cowpea (*Vigna unguiculata* L.)

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### Abstract

Anthracnose caused by *Colletotrichum lindemuthianum* is a major disease, lead to substantial yield loss of cowpea. Pink-pigmented facultative methylotrophs are a group of aerobic facultative methylotrophic, Gram negative, rod shaped bacteria comes under the genus *Methylobacterium*. They colonise the phyllosphere and rhizosphere of many plants, interact with them, thus promote growth and suppress plant diseases. This study aimed to isolate PPFMs from cowpea phyllosphere, identify them morphologically and molecularly, and evaluate their biocontrol potential against anthracnose pathogen *C. lindemuthianum*. Isolation was carried out by leaf imprint method on ammonium-mineral salts medium supplemented with 0.5% methanol. Typical pink coloured colonies were observed five to seven days after inoculation. Selected 10 PPFM isolates were subjected for biochemical, morphological and molecular characterization via 16S rRNA gene sequencing. *in vitro* antagonism studies showed that PPFM 8 has strong antagonistic potential, inhibited mycelial growth of *C. lindemuthianum* with 58.27% inhibition. Further, 16S rRNA gene sequence showing 99.93% identity with *Methylobacterium populi*. Comparative evaluation of PPFM isolate and biocontrol agents against *C. lindemuthianum* revealed that *T. asperellum* significantly inhibited the radial mycelial growth followed by PPFM 8 with 58.65% inhibition. These findings demonstrate that PPFMs are promising candidates for sustainable management of anthracnose in cowpea, warranting further field validation and formulation development

**Keywords:** *Methylobacterium*, *C. lindemuthianum*, PPFMs, cowpea, biocontrol

### Introduction

Cowpea (*Vigna unguiculata* L.) is a key legume crop in tropical and subtropical regions, valued for its protein-rich seeds, drought tolerance and suitability in marginal soils. It is widely used as dal, vegetable and fodder. Cowpea seeds hold significant importance in the dietary regimes of both rural and urban populations, serving as an alternative source of protein to that derived from animal sources (Wakili, 2013) [45]. The crop also serves as a source of livestock protein (Owolade *et al.*, 2006) [36].

Fungi, bacteria, viruses and nematodes are the important pathogenic organisms associated with cowpea diseases (Emechebe and Lagoke, 2002) [7]. Anthracnose disease caused by the hemibiotrophic fungus *C. lindemuthianum* is one of the major foliar and pod diseases of cowpea, causing significant yield losses under warm and humid conditions. Numerous species complexes of the genus *Colletotrichum*, including *C. acutatum* (lupin species), *C. truncatum* (lentils and soybeans), *C. destructivum* (clover, alfalfa, cowpea, and lentil), *C. orchidearum*, *C. magnum* (soybean), *C. dematium* (cowpea), *C. spaethianum* (common bean), *C. gloeosporioides* (cowpea and beans), *C. chlorophyti* (soybean), and *C. coccodes* (soybean), are known to infect legumes on a global scale (Damn *et al.*, 2014) [5]. Anthracnose infection to a susceptible cultivar under conducive conditions could result in a 100 % yield loss (Fernandez *et al.*, 2000) [9].

It infects whole above ground plant parts such as leaves (Emechebe and Lagoke, 2002) [7], stem, pods and fruits (Akinode and Ikotun, 2008) [2]. This fungus persists in crop debris, and transmitted through seeds as dormant mycelium associated with seed coat or as spores present between the cotyledons; from where it initiates infection of hypocotyls and young leaves in the field. The symptom appear as small dark brown to black lesions on leaves. These tissues later exhibits small rust coloured specks, gradually enlarge to form sunken lesions (Falade, 2016) [8]. Fungus forms microsclerotia, survive in soil for extended periods and are resistant to environmental stresses. In tropical and subtropical regions, this disease results in significant losses. Conventional control methods of anthracnose of cowpea rely on chemical fungicides, which pose environmental, health and resistance development concerns. Combination of different biological agents has demonstrated significant potential in the control of anthracnose of leguminous vegetables (Kumar *et al.*, 2020) [23]. There is increasing interest in biological control and plant-associated beneficial microorganisms.

Pink-pigmented facultative methylotrophs (PPFMs) are aerobic Gram-negative bacteria, typically of the genus *Methylobacterium*, utilize single-carbon compounds like methanol released from plant tissue, and mainly colonise on the phyllosphere of many plants. They can grow also on a variety of C<sub>2</sub>, C<sub>3</sub> and C<sub>4</sub> compounds (Ivanova *et al.* 2001) [16]. Several species of methylotrophic bacteria are commonly associated with both terrestrial and aquatic plants, where they colonize roots, leaf surfaces, and developing buds (Lidstrom and Chistoserdova, 2002) [28]. They are recognised for plant-growth-promoting traits such as phytohormone production (Holland, 1997) [13], nitrogen fixation and nodule formation (Raja *et al.* 2006), phosphate

solubilisation (Jayashree *et al.*, 2011b) [19, 20], synthesis of siderophores (Simionato *et al.* 2006) [42], ACC-deaminase activity (Madhaiyan *et al.*, 2006) [30] and biocontrol activity against pathogens (Kumar *et al.*, 2015) [24]. PPFMs demonstrates biocontrol potential against phytopathogens through multiple mechanisms, including the production and secretion of antibiotics, siderophores, secondary metabolites, hydrogen cyanide, etc. (Koshy *et al.*, 2025) [22]. They enhance defence reaction in plants by synthesize and release of PR (pathogenesis-related) proteins as well as defense and antioxidant enzymes like  $\beta$ -1,3-glucanase, LOX (lipoxygenase), chitinase, PO (peroxidase), PAL (phenylalanine ammonia lyase), CAT (catalase) and PPO (polyphenol oxidase) (Janahiraman *et al.*, 2016) [17]. PPFMs have shown antagonistic effects against various fungi including *Colletotrichum* spp. and *Rhizoctonia* spp. (e.g., Nysanth *et al.*, 2023; Poorniammal *et al.*, 2009) [34, 37].

Given this, the present study was conducted with the following objectives: (i) isolate and identify PPFMs from cowpea phyllosphere, (ii) screen these isolates for antagonistic activity against *C. lindemuthianum* *in vitro*. The aim of this study is to evaluate the feasibility of using PPFMs as biocontrol agents against anthracnose of cowpea.

## Materials and Methods

### Sampling and isolation of PPFMs

Leaf samples were collected from healthy cowpea plants growing in five agro-ecological units (AEUs) of Kerala including AEU 2, AEU 7, AEU 11, AEU 20, and AEU 21, covering Kasaragod, Kannur, Kozhikode, and Wayanad districts of Kerala (Table 1). The healthy leaves were collected and placed in sterile polythene bags. The leaf samples were stored in a refrigerator at 4 °C until further isolation.

**Table 1;** Details of sample and PPFM isolates used for the study

| Location of sample collection | AEUs (Agro-Ecological Units) |                         | Latitude/longitude    | Variety    | Code of the isolates |
|-------------------------------|------------------------------|-------------------------|-----------------------|------------|----------------------|
| Ambalavayal, Wayanad          | AEU-20                       | Wayanad Central Plateau | 11°38'08"N/76°12'37"E | Lola       | PPFM 1               |
| Ambalavayal, Wayanad          | AEU-20                       | Wayanad Central Plateau | 11°38'08"N/76°12'37"E | Lola       | PPFM 2               |
| Ulliyeri, Kozhikode           | AEU-11                       | Northern laterites      | 11°26'00"N/75°47'00"E | Lola       | PPFM 3               |
| Noolpuzha, Wayanad            | AEU-21                       | Wayanad Eastern Plateau | 11°40'00"N 76°18'50"E | Hybrid     | PPFM 4               |
| Dharnadom, Kannur             | AEU-2                        | Northern Coastal Plains | 11°47'36"N/75°28'15"E | Lola       | PPFM 5               |
| Kannapuram, Kannur            | AEU-7                        | Kaipad Lands            | 11°58'09"N/75°19'15"E | Vyjayanthi | PPFM 6               |
| Madayi, Kannur                | AEU-2                        | Northern Coastal Plains | 11°58'00"N/75°18'00"E | Hybrid     | PPFM 7               |
| Mangattidom, Kannur           | AEU-11                       | Northern laterites      | 11°51'40"N/75°33'45"E | Lola       | PPFM 8               |
| Pilikode, Kasargod            | AEU-7                        | Kaipad Lands            | 12°11'25"N/75°10'0"E  | Hybrid     | PPFM 9               |
| Kayyur-Cheemeni, Kasargod     | AEU-11                       | Northern laterites      | 12°24'25"N/75°18'0"E  | Githika    | PPFM 10              |

### Isolation and purification of PPFMs

PPFMs were isolated by following leaf imprinting technique in Ammonium mineral salt agar (AMS) medium (Lidstrom and Chistoserdova, 2002) [28] supplemented with 0.5% (v/v) methanol and filter sterilized cycloheximide (50 µg ml<sup>-1</sup>). Collected leaves were washed in running tap water, dried at room temperature to remove the moisture, gently pressed for 2 minutes onto AMS agar medium plated in sterile Petri dish. The leaves were lifted out and the plates were incubated in an inverted position at 28 ± 2 °C for five to seven days. Characteristic pink colonies appeared on the medium 5 to 7 days after inoculation. Pink coloured single colonies were selected and purified by quadrant streaking. Purified bacterial isolates were maintained in AMS slants in a refrigerator at a temperature of 4 °C and used for further studies.

### Morphological and biochemical characterization of PPFMs

The isolates were characterized based on colony morphology (colour, size, and margin), Gram reaction, cell shape, motility, and enzymatic activities such as catalase and oxidase tests for preliminary identification. Morphological characters such as pigmentation, shape and size of the colony, Gram reaction, motility etc. were studied after growing them in AMS agar medium for 7 days at 28 ± 2 °C. Bacterial cells were stained with methylene blue and observed under a microscope to determine the cell shape (Becking, 1974) [3]. Motility of the PPFM isolates was examined by hanging drop method using a cavity slide. Biochemical test such as ortho-nitrophenyl- $\beta$ -galactoside, lysine utilization, ornithine utilization, urease, phenyl alanine deamination, nitrate reductase, hydrogen sulphide

production, citrate utilization, Voges Proskauer's (VP), methyl red (MR), indole, malonate utilization, esculin hydrolysis tests were carried out using HiMedia (Hi25™ Enterobacteriaceae) bacterial identification kit. Each well in the strips was inoculated with 50 µl bacterial suspension with  $10^8$  cfu ml<sup>-1</sup> and were incubated at 28 °C for 24 h. Observations were recorded by using proper reagents contained in the kit. Tests like oxidase was carried out using oxidase disk. Biochemical test for catalase was performed by the protocols given by Khatoon *et al.* (2022) [21].

### Isolation of *C. lindemuthianum*

Cowpea leaves exhibiting anthracnose symptoms were collected from farmer's field. The leaves were thoroughly washed in running tap water. Small leaf bits (2-5 mm) with healthy and infected portion were taken and immersed into one Percent sodium hypochlorite for one minute for surface sterilization. Leaf pieces were washed in three changes of sterile distilled water and air dried at room temperature. Three leaf pieces were placed on to a Petri plate containing potato dextrose agar (PDA). Inoculated plates were kept for incubation at  $28^0 \pm 2$  °C for 7 days. Single spore from a developing colony was isolated and sub cultured for purification. The purified fungal culture was stored in PDA slants at 4 °C for further study. Pathogenicity was established by artificially inoculating healthy cowpea plants with the cultured fungal isolate and observing the development of characteristic anthracnose symptoms.

### *In vitro* evaluation of antagonistic effect of PPFM isolates against *C. lindemuthianum* by dual culture technique

Dual culture technique (Dennis and Webster, 1971) [6] was employed for *in vitro* antagonism studies to study the efficiency of PPFM isolates against the anthracnose pathogen. A virulent isolate of *C. lindemuthianum* obtained from anthracnose affected cowpea and maintained on PDA. A 5 mm agar plug of *C. lindemuthianum* taken from 7 days old actively growing culture was placed at the centre of sterile Petri plates containing PDA. PPFM isolate streaked 2.5 cm away from plug on two opposite sides. The plates were incubated at  $28^0 \pm 2$  °C for seven days. Three replications were maintained along with a control plate devoid of PPFM. The radial growth of the fungal pathogen was recorded when it reached full growth in the control plate, and the percentage inhibition was calculated related to the pathogen growth in the control (Vincent, 1947) [44].

$$\text{Percent Growth Inhibition (P.I)} = \frac{C - T}{C} \times 100$$

Where C is the growth of the pathogen in control and T is the growth of pathogen in dual culture plate.

### Comparative evaluation of antagonistic effect of selected strains of PPFMs and biocontrol agents against *C. lindemuthianum* under *in vitro* conditions

Antagonistic effect of two selected strains of PPFMs was compared with biocontrol agents like *Trichoderma asperellum* ( $10^6$  cfu ml<sup>-1</sup>) and *Pseudomonas fluorescens* ( $10^8$  cfu ml<sup>-1</sup>) by dual culture method (Dennis and Webster, 1971) [6]. Autoclaved molten PDA at equal was poured into sterile Petri plates. PPFM isolates and *P. fluorescens* were streaked parallel on one side of the Petri plates 2 cm away from the periphery and the fungal pathogen disc taken from

7 days old culture was placed on the other side 2 cm from periphery. For the comparative study with *T. asperellum*, sterile molten PDA was poured into Petri plates, and after solidification 5 mm mycelial discs of *T. asperellum* and *C. lindemuthianum* were taken from seven-days old actively growing cultures placed on opposite sides of the plate, each positioned 2 cm from the periphery. The Petri plate inoculated only with pathogen was maintained as control. Inoculated plates were kept for incubation at  $28^0 \pm 2$  °C for seven days. The experiment was laid out following CRD (completely randomized design) with three replications. The Percent inhibition of mycelial growth was calculated as per the formula described above.

### Molecular characterization of PPFM isolate

The best PPFM isolate with maximum Percent inhibition to *C. lindemuthianum* was selected and subjected for molecular characterization. Molecular characterization of selected PPFM was carried out by 16S rRNA sequencing. PCR amplification was performed using 16S rRNA universal primers (forward primer: 27F/1492R) in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR conditions were initial denaturation 95 °C 5 min followed by 35 cycles of 94 °C 45s, 52 °C 60 s, 72 °C 90 s and final extension 72 °C 10 min. for amplification of 16S rRNA genes. The PCR product was sequenced with an ABI 3500 DNA Analyzer (Applied Biosystems) using the Sanger DNA sequencing method. The resulting sequences were aligned and edited using Geneious Pro v5.1. Sequence similarity was assessed via the Basic Local Alignment Search Tool (BLAST). The assembled contigs were compared against 16S rRNA gene sequences of bacterial isolates in the NCBI Nucleotide database, and the isolates were identified based on the highest percentage sequence similarity.

Phylogenetic tree was constructed by neighbourhood joining method, MEGA X to confirm placement within *Methylobacterium* genus. Bootstrap values were computed utilizing 1000 replicates in conjunction with the Kimura 2-parameter model and six gamma categories to accurately represent the variation in substitution rates among sites.

### Statistical analysis

*In vitro* experiments were conducted using completely randomized design (CRD) with three replications. All data were subjected to analysis of variance (ANOVA) using Web Agri Stat (WASP) package.

## Results and Discussion

### Isolation and preliminary characterisation

A total of 10 PPFM isolates were obtained from phyllosphere of cowpea collected from major cowpea growing areas of Kerala comprising five AEUs (AEU 2, 7, 11, 20 and 21) covering Kozhikode, Wayanad, Kannur and Kasargod districts. Convex to slightly convex and round colonies showed typical medium pink to light pink pigmentation with entire and undulate margin (Fig. 1). Microscopically, the cells were Gram-negative rods and motile (Table 2). The majority of the morphological characters of 10 PPFM isolates were similar to other *Methylobacterium* spp.



**Fig 1:** Isolation, purification and maintenance of PPFMs from cowpea leaves by leaf imprinting method

**Table 2:** Morphological characters of PPFM isolates on AMS medium

| Sl. No. | PPFM isolate | Colony shape | Margin of the colony | Elevation of the colony | Pigmentation | Cell shape | Motility | Gram reaction |
|---------|--------------|--------------|----------------------|-------------------------|--------------|------------|----------|---------------|
| 1       | PPFM 1       | Round        | Entire               | Slightly convex         | Light pink   | Rod        | Motile   | Gram-negative |
| 2       | PPFM 2       | Round        | Entire               | Slightly convex         | Light pink   | Rod        | Motile   | Gram-negative |
| 3       | PPFM 3       | Round        | Entire               | Slightly convex         | Light pink   | Rod        | Motile   | Gram-negative |
| 4       | PPFM 4       | Irregular    | Undulate             | Slightly convex         | Light pink   | Rod        | Motile   | Gram-negative |
| 5       | PPFM 5       | Irregular    | Entire               | Flat                    | Light pink   | Rod        | Motile   | Gram-negative |
| 6       | PPFM 6       | Irregular    | Entire               | Flat                    | Light pink   | Rod        | Motile   | Gram-negative |
| 7       | PPFM 7       | Round        | Entire               | Slightly convex         | Light pink   | Rod        | Motile   | Gram-negative |
| 8       | PPFM 8       | Round        | Entire               | Convex                  | Medium pink  | Rod        | Motile   | Gram-negative |
| 9       | PPFM 9       | Round        | Entire               | Flat                    | Light pink   | Rod        | Motile   | Gram-negative |
| 10      | PPFM 10      | Round        | Entire               | Slightly convex         | Pink         | Rod        | Motile   | Gram-negative |

### Biochemical characterization of PPFMs

Biochemical characterization of ten PPFM isolates revealed a high degree of uniformity in several biochemical reactions (Table 3). All PPFM isolates were positive for oxidase, catalase, urease, citrate utilization, indole and MR tests. All isolates were negative for ONPG, VP test, and phenylalanine deamination. Variation was observed in a few tests. Nitrate reduction was positive in seven isolates (PPFM

1 to 7) but negative in PPFM 8, 9, and 10. H<sub>2</sub>S production was noticed in six isolates, while four isolates were negative. Lysine utilization was observed only in PPFM 9. Ornithine utilization was positive in PPFM 3, 4, 6, 9, and 10. Malonate utilization was positive in PPFM 2, 7, and 8. Esculin hydrolysis was positive in all isolates except PPFM 10.

**Table 3:** Biochemical characterization of PPFMs

| Biochemical tests              | PPFM 1 | PPFM 2 | PPFM 3 | PPFM 4 | PPFM 5 | PPFM 6 | PPFM 7 | PPFM 8 | PPFM 9 | PPFM 10 |
|--------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|
| Oxidase                        | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| Catalase                       | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| Urease                         | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| Citrate utilization            | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| Indole production              | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| MR test                        | +      | +      | +      | +      | +      | +      | +      | +      | +      | +       |
| Orto-Nitrophenyl-β-galactoside | -      | -      | -      | -      | -      | -      | -      | -      | -      | -       |
| Nitrate reduction              | +      | +      | +      | +      | +      | +      | +      | -      | -      | -       |
| H <sub>2</sub> S production    | +      | +      | +      | -      | -      | -      | +      | +      | +      | -       |
| VP test                        | -      | -      | -      | -      | -      | -      | -      | -      | -      | -       |
| Phenylalanine deamination      | -      | -      | -      | -      | -      | -      | -      | -      | -      | -       |
| Lysine utilization             | -      | -      | -      | -      | -      | -      | -      | -      | +      | -       |
| Ornithine utilization          | -      | -      | +      | +      | -      | +      | -      | -      | +      | +       |
| Malonate utilization           | -      | +      | -      | -      | -      | -      | +      | +      | -      | -       |
| Esculin hydrolysis             | +      | +      | +      | +      | +      | +      | +      | +      | +      | -       |

### In vitro evaluation of antagonistic effect of PPFMs against *C. lindemuthianum* by dual culture technique

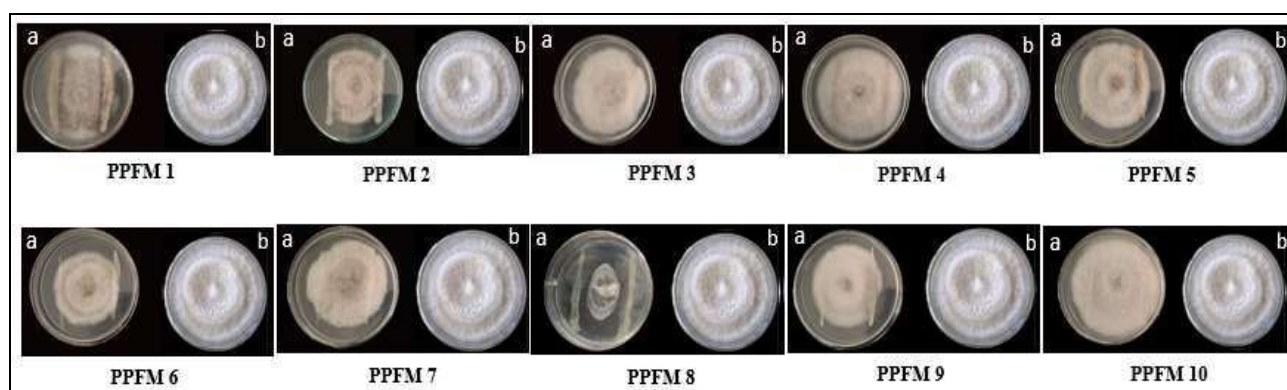
The antagonism effect of 10 PPFM isolates against the test pathogen was evaluated through dual culture assay, and the results revealed significant variation among the isolates (Table 4). The mean radial growth of *C. lindemuthianum* in dual culture was ranged from 3 cm to 9 cm compared to 9 cm in control. In dual culture assay, all isolates except

PPFM 4 and PPFM 10 exhibited direct antagonism against *C. lindemuthianum*. PPFM 8 significantly inhibited mycelial growth of *C. lindemuthianum* with maximum inhibition (58.27%) followed by PPFM 2 (34.43%). PPFM 6 showed the least inhibitory effect (18.51%). Statistical analysis indicated significant differences among treatments, confirming that some PPFM isolates possess good antagonistic activity against *C. lindemuthianum* (Fig.2).

**Table 4:** Percent mycelial growth inhibition of *C. lindemuthianum* on PDA by PPFM isolates, seven days after inoculation

| Sl. No.  | PPFM isolates | Colony diameter of pathogen in control (cm) | Mean growth of the pathogen in dual culture (cm) | Percent growth inhibition (%) <sup>*</sup> |
|----------|---------------|---------------------------------------------|--------------------------------------------------|--------------------------------------------|
| 1        | PPFM 1        | 9                                           | 7.16 <sup>bc</sup>                               | 20.36(26.77) <sup>b</sup>                  |
| 2        | PPFM 2        | 9                                           | 5.9 <sup>cd</sup>                                | 34.43(34.11) <sup>b</sup>                  |
| 3        | PPFM 3        | 9                                           | 6.7 <sup>bc</sup>                                | 24.81(29.71) <sup>b</sup>                  |
| 4        | PPFM 4        | 9                                           | 9.0 <sup>a</sup>                                 | 0(0.29) <sup>c</sup>                       |
| 5        | PPFM 5        | 9                                           | 7.3 <sup>b</sup>                                 | 18.51(25.43) <sup>b</sup>                  |
| 6        | PPFM 6        | 9                                           | 7.3 <sup>b</sup>                                 | 18.51(25.12) <sup>b</sup>                  |
| 7        | PPFM 7        | 9                                           | 7.5 <sup>b</sup>                                 | 29.62(32.40) <sup>b</sup>                  |
| 8        | PPFM 8        | 9                                           | 4.8 <sup>d</sup>                                 | 58.27(49.76) <sup>a</sup>                  |
| 9        | PPFM 9        | 9                                           | 6.9 <sup>bc</sup>                                | 22.59(28.31) <sup>b</sup>                  |
| 10       | PPFM 10       | 9                                           | 9.0 <sup>a</sup>                                 | 0(0.29) <sup>c</sup>                       |
| CD(0.05) |               |                                             | 1.385                                            | 18.036                                     |

<sup>\*</sup>Data are presented as the means  $\pm$ SE of three replications. In each column figure followed by same letter do not differ significantly according to DMRT. Arc sine transformed values are given in parenthesis.

**Fig 2:** Antagonistic effect of PPFMs against *C. lindemuthianum* by dual culture plate assay. (a) PPFM isolate and test pathogen (b) Control plate-*C. lindemuthianum* only.

#### Comparative evaluation of antagonistic effect of selected strains of PPFMs and biocontrol agents against *C. lindemuthianum* under *in vitro* conditions

Dual culture method was followed for the comparative evaluation of antagonistic activity of PPFM isolates with *T. asperellum* and *P. fluorescens* against the pathogen *C. lindemuthianum*. In dual culture assay there was no zone of inhibition by *T. asperellum* against *C. lindemuthianum* but antagonism was visible as rapid growth and direct

mycoparasitism interaction Fig. 3. A clear zone of inhibition between the two organisms was appeared in case of *P. fluorescens*. Under *in vitro* conditions, *T. asperellum* exhibited the highest antagonistic activity against *C. lindemuthianum* with 66.66% inhibition, followed by PPFM 8 with 58.65% inhibition. *P. fluorescens* showed a comparatively lower level of inhibition, suppressing the pathogen by only 41.10% (Table 5).

**Table 5:** *In vitro* evaluation of selected PPFM isolates and biocontrol agents against *C. lindemuthianum*

| Sl. No.   | Treatments            | Colony diameter of pathogen (cm) | Percent growth inhibition (%) <sup>*</sup> |
|-----------|-----------------------|----------------------------------|--------------------------------------------|
| 1         | PPFM 2                | 6.0 <sup>a</sup>                 | 31.10(33.90) <sup>d</sup>                  |
| 2         | PPFM 8                | 4.26 <sup>c</sup>                | 58.65(50.10) <sup>b</sup>                  |
| 3         | <i>P. fluorescens</i> | 5.16 <sup>b</sup>                | 41.10(39.80) <sup>c</sup>                  |
| 4         | <i>T. asperellum</i>  | 3.0 <sup>d</sup>                 | 66.66(54.74) <sup>a</sup>                  |
| CD (0.05) |                       | 0.277                            | 4.97                                       |

<sup>\*</sup>Data are presented as the means  $\pm$ SE of three replications. In each column figure followed by same letter do not differ significantly according to DMRT. Arc sine transformed values are given in parenthesis.

**Fig 3:** Comparative evaluation of antagonistic effect of selected strains of PPFMs and biocontrol agents against *C. lindemuthianum* under *in vitro* conditions. a. Control (*C. lindemuthianum*) and *C. lindemuthianum* x PPFM 2, b. Control (*C. lindemuthianum*) and *C. lindemuthianum* x PPFM 8, c. Control (*C. lindemuthianum*) and *C. lindemuthianum* x *P. fluorescens*, d. Control (*C. lindemuthianum*) and *C. lindemuthianum* x *T. asperellum*.

### Molecular characterization of selected PPFM isolate

Genomic DNA was extracted from PPFM 8, and the polymerase chain reaction amplified 16S rRNA genes were subsequently purified. The 16S rRNA gene from the isolate PPFM 8 was sequenced and compared the sequence with the NCBI GeneBank database. The isolate, PPFM 8 confirmed

as *Methylobacterium populi*, showed 99.93% identity with *M. populi*. Phylogenetic tree placed them within the *Methylobacterium* clade with bootstrap support of more than 90%. The sequence was deposited in GenBank as accession number PX716687. The taxonomy and methylotrophic trait are consistent with PPFM identity (Table 7).

**Table 7:** Identification of PPFM isolate by 16S rRNA sequencing

| PPFM isolate | Gene bank accession No. | Most closely related organism |                                                                             |                           |                            |
|--------------|-------------------------|-------------------------------|-----------------------------------------------------------------------------|---------------------------|----------------------------|
|              |                         | Standard species              | Accession description*                                                      | Percent gene identity (%) | Percent query coverage (%) |
| PPFM 8       | PX716687                | <i>M. populi</i>              | <i>Methylobacterium populi</i> strain L3-216 16S rRNA gene partial sequence | 99.93                     | 95                         |

\* Data obtained after BLAST analysis from NCBI database

The phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolate PPFM 8 (Accession No. PX716687) clustered closely with *Methylobacterium populi* (JQ659337.1), forming a well-supported clade with a high bootstrap value of 99.93%, indicating a strong phylogenetic relationship. This clade was again grouped with *M. lusitanum* and *M. zzatmanii*, supported by bootstrap values of 98 and 94% respectively, revealed close evolutionary relatedness among these taxa (Fig. 4).

The phylogenetic tree clearly separated members of the genus *Methylobacterium* from other *Methylobacterium* species,

with *M. extorquens*, *M. suomiense*, and *M. salsuginis* forming distinct lineages. Another well supported cluster comprised *M. nodulans* and *Methylobacterium* sp. (DQ838527.1), supported by a bootstrap value of 100%. Similarly, *M. mesophilicum*, *M. radiotolerans*, and *M. fujisawaense* formed a separate clade with strong bootstrap support ranging from 82 to 100%. The phylogenetic placement confirms that isolate PX716687 belongs to the genus *Methylobacterium* and is most closely related to *Methylobacterium populi*, thereby validating its taxonomic affiliation based on molecular evidence.

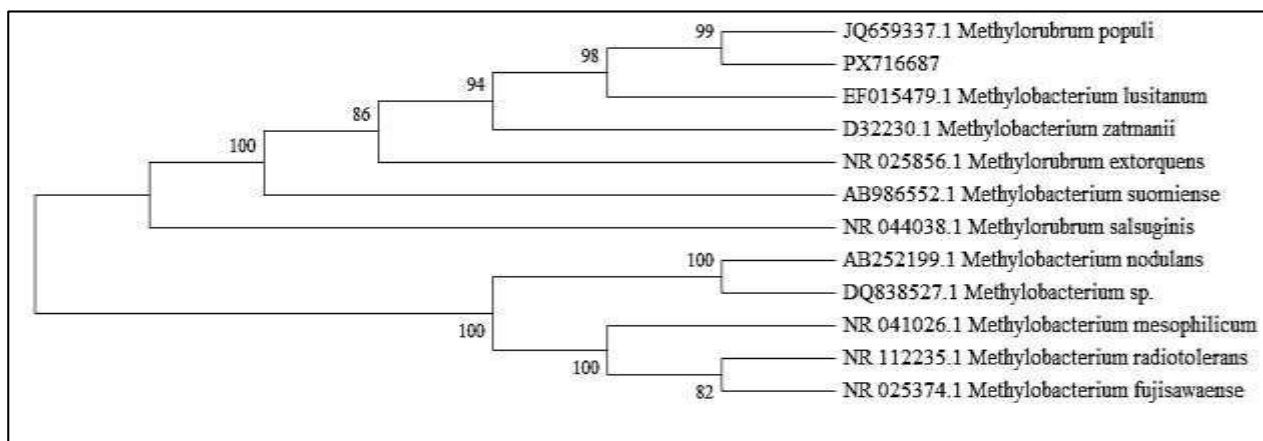


**Fig 4:** Phylogenetic tree showing the relationship of isolate PPFM 8 (GenBank Accession No. PX716687) with other *Methylobacterium* spp. and *Methylobacterium* spp. using Neighbourhood joining method. The bootstrap consensus tree inferred from 1000 replicates.

### Discussion

This study demonstrates that PPFMs isolated from cowpea phyllosphere, tentatively identified as methylotrophic bacterium by its pink pigmented, convex to slightly convex and round colonies with entire and undulate margin on AMS medium supplemented with methanol as the sole source of carbon and energy. They have the ability to utilize methanol, a cell wall pectin breakdown product from plants with the help of methanol dehydrogenase enzyme. AMS medium supplemented with 0.5% methanol is the minimal medium used to isolate PPFMs from different plant parts (Holland *et al.*, 2000) [14]. Several authors have previously reported the natural association of PPFMs with phyllosphere of many plants. All isolates were proven to be methylotrophic since they grew in carbon limited medium. The isolation method using AMS with 0.5% methanol effectively recovered PPFM bacteria, in agreement with prior work on PPFMs from other crop plants (Nysanth *et al.* 2023) [34]. Previous investigations found that several species of *Methylobacterium* genus were isolated from a variety of

plants, such as rice, potato, banana, soybeans, and oil palm (Ishak *et al.*, 2021; Senthilkumar *et al.*, 2021; Christian *et al.*, 2021; Grossi *et al.*, 2020; Lai *et al.*, 2020) [15, 41, 4, 12, 26]. Morphological characters of PPFM isolates suggest that all isolates yielded pink pigmented colonies with Gram negative, rod shaped and motile cells. Colony morphology was varied with different isolates. PPFMs isolated from the phyllosphere of neem (*Azadirachta indica*) exhibited pink to orange-pink pigmentation, entire margin, round colonies with a smooth glistening surface, and convex elevation (Kumar and Lee, 2009) [25]. Previous studies have also reported differences in the colony morphology of PPFM bacteria isolated from different sources (Jang and Lee 2008; Lo and Lee, 2007) [18, 29]. Characteristic medium pink-coloured colonies with Gram-negative, rod-shaped, motile cells were isolated from the rice phyllosphere and identified as *M. populi* (Nysanth *et al.*, 2018) [35]. The result of the present study are in confirmation with the findings of Raghavendra and Santhosh (2019) [38] reported the isolation of 50 PPFMs from the phyllosphere and rhizosphere of

direct seeded rice, yielded characteristic pink pigmented colonies. The morphological traits observed are in align with the established characteristics of the genus *Methylobacterium*, with the most significant feature being the presence of reddish-pink pigmentation of colonies in AMS medium.

Biochemical characterisation of PPFM isolates supports their identification as PPFMs by showing a consistent profile across various tests. All isolates were positive for catalase, oxidase, urease, citrate, indole production and MR tests. Positive result for indole production and MR tests indicate the presence of stable metabolic pathways associated with tryptophan degradation. Variation among the isolates were noticed in nitrate reduction, H<sub>2</sub>S production, amino acid utilization and malonate utilization, reflecting intraspecific diversity commonly reported within PPFMs. The findings of the present study are supported by the findings of Jang and Lee (2008) [18], obtained positive result for amylase, catalase, oxidase and urease for all PPFMs isolated from soil. Five PPFM isolates cultured from paddy were positive for oxidase, catalase, urease and indole production (Nysanth *et al.*, 2018) [35]. These findings are also consistent with the results of Ramli *et al.* (2024) [39], gained catalase, indole, H<sub>2</sub>S production, urease and citrate positive reactions in PPFMs isolated from Malaysian ulam leaves. The biochemical profiles obtained in this study are consistent with previously documented characteristics of *Methylobacterium* spp.

In this study 10 PPFMs were isolated from cowpea grown across five AEUs (AEU 2, 7, 11, 20 and AEU 21) of Kerala. The biocontrol efficiency of the isolates was tested by dual culture assay. Among different PPFM isolates, PPFM 8 exhibited higher antagonistic potential (58.27%) against the anthracnose fungus, *C. lindemuthianum*. The antagonistic effect of PPFM in the current study in the dual culture assay may be related to some diffusible antifungal metabolites of the PPFM isolate, revealed its strong biocontrol efficiency against the test pathogen. Similar results have been reported in earlier investigations. *Methylobacterium* spp. significantly reduced the linear mycelial growth of *Fusarium udum*, *F. oxysporum*, *Pythium aphanidermatum* and *Sclerotium rolfsii* under *in vitro* conditions (Janahiraman *et al.*, 2016) [17]. The PPFM isolates AM1 and CO-47 were found to produce highly effective volatile antibiotics against *F. oxysporum* and *F. udum* *in vitro* (Poorniammal *et al.*, 2009) [37]. PPFMs have been reported to exhibit biocontrol efficiency to inhibit plant pathogens such as *Xanthomonas campestris*, *S. rolfsii*, *F. oxysporum*, *C. capsici* and *Cercospora capsici*, as evidenced by the zone of inhibition (Gamit *et al.*, 2023) [10]. PPFMs released some volatile organic compounds (Naznin *et al.*, 2013) [32] which induce ISR in plants along with the production of cell wall degrading enzymes such as pectinase, cellulases and hemicellulases, and glucosidases (Lee *et al.*, 2006; Madhaiyan *et al.*, 2006) [27, 30]. PPFM isolated from amaranthus leaves identified as *M. hispanicum* exhibited 53.88% inhibition to *R. solani* under *in vitro* conditions (Nambiar, 2023) [31]. PPFMs enhance plant's defence mechanism by inducing the production of defence enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO), as well as by stimulating the synthesis of pathogenesis-related (PR) proteins like  $\beta$ -1,3-glucanase and chitinase (Nysanth *et al.*, 2023) [34]. The multi-mechanistic nature of PPFM action

along with growth promotion and biocontrol makes them attractive for integrated disease management, especially for legume crops like cowpea.

Comparative evaluation of PPFM isolates, *T. asperellum* and *P. fluorescens* revealed that the antagonistic efficiency varied significantly with biocontrol agents. Among the treatments, *T. asperellum* was proved to be the most effective, exhibiting 66.66% inhibition of *C. lindemuthianum*. The strong inhibitory effect of *T. asperellum* may be attributed to its biocontrol mechanisms such as mycoparasitism, competition for nutrients and space, and the production of antifungal metabolites and cell wall degrading enzymes. Similar findings have been reported in earlier studies where *Trichoderma* sp. demonstrated superior antagonism against various fungal pathogens due to their aggressive growth and production of lytic enzymes. *T. asperellum* advanced toward the mycelium of *C. lindemuthianum* and eventually overgrew it (Ajayi and Oyedele, 2016). Under *in vitro* conditions, *T. viride* recorded the highest mycelial growth inhibition of *C. lindemuthianum* (69.44%), followed by *T. harzianum* (63.88%) (Wani *et al.*, 2024) [46].

Among the bacterial antagonists, PPFM 8 exhibited superior efficacy compared to PPFM 2 and *P. fluorescens*, resulting in 58.65% inhibition of mycelial growth, indicating its potential role as an effective antagonist. The superior effect of PPFM 8 may be due to the synthesis of bioactive compounds, siderophores, and phytohormones, as well as its ability to efficiently colonize the growth medium and compete with the pathogen. PPFM isolate IISRGPPFM 38, obtained from ginger phyllosphere exhibited 50 to 65% inhibition against *C. lindemuthianum* under *in vitro* conditions (Vadivukkarasi and Bhairi, 2020) [43]. PPFMs have the ability to synthesize enzymes like, cellulase, pectinase, lipase, amylase and chitinase as well as production of siderophores as the indication of biocontrol efficiency. Many researchers have reported that this bacteria can produce growth hormones and mycolytic enzymes, which play a role in resistance mechanisms (Jayashree *et al.*, 2011a) [19, 20]. The findings disclose that *T. asperellum* and PPFM 8 possess strong antagonistic effect against *C. lindemuthianum* and could be further evolved as potential biocontrol agent.

Phylogenetic analysis confirms that the studied isolate (PPFM 8) belongs to the genus *Methylobacterium* and shows closest similarity to *M. populi*, supported by high bootstrap value. As reported by Green and Ardley (2018) [11], certain species previously classified under *Methylobacterium* have been reallocated to a new genus, *Methylobacterium* based on some dissimilarities. Most *Methylobacterium* species can utilize methylamine as the sole source of carbon and energy, whereas the majority of *Methylobacterium* species lack this ability.

Compared to chemical control, PPFMs offer environmentally friendly alternatives, potentially reducing fungicide usage and enhancing plant growth simultaneously. However, field-level trials across locations and seasons are needed to validate the findings and to assess formulation, application timing and compatibility with other inputs.

## Conclusion

The study explained the presence of PPFMs in the leaves of cowpea, possess potent biocontrol activity against *C. lindemuthianum* causing anthracnose disease. The

morphological, biochemical and molecular characteristics revealed that they can be assigned as PPFMs. Specifically, isolates such as PPFM 8 (identified as *M. populi*) showed strong antagonism against the test pathogen under *in vitro* conditions. Comparative study with *T. asperellum* and *P. fluorescens* showed PPFM possess potent antagonistic effect against *C. lindemuthianum*. These results support the potential of PPFMs as biocontrol agents in cowpea production systems. Hence, PPFM could be an efficient bioagent for disease management and growth improvement of cowpea. Future work should focus on field validation, formulation development, metabolite identification and integration into crop management packages.

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### Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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