Indian J. Applied & Pure Bio. Vol. 41(1), 38-45 (2026). **ISSN: 0970-2091**

Assessment of Abiotic Stress tolerance Traits in Pink Pigmented Facultative Methylotrophic Bacteria (PPFM) Isolated from Rice

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Abstract

Salinity, drought, and extreme temperature are the abiotic stresses that significantly affect agricultural productivity. Pink Pigmented Facultative Methylotrophs (PPFM) are plant growth-promoting bacteria mainly associated with the phyllosphere region, which have been documented to have stress-tolerant capacity. In this study, five PPFM isolates were obtained from the phyllosphere and rhizosphere of different rice cultivars in Cuddalore district, Tamil Nadu. The isolates were characterized based on morphological and biochemical characters. Subsequently, they were evaluated in vitro for their tolerance to salt (up to 3% NaCl), drought (5-30% using PEG 6000) and temperature (45°C–55°C) stresses. Among the isolates, PPFM 1, obtained from the phyllosphere of the ADT 47 rice cultivar, demonstrated superior tolerance across all stress conditions. It maintained the highest cell viability at 3% NaCl (2.41 Log CFU/ml), 55°C (0.98 Log CFU/ml), and 15% PEG (0.25) with a measurable optical density. The isolate PPFM 1 was molecularly identified as *Methylobacterium phyllosphaerae*. This study suggests that indigenous PPFM isolates have abiotic stress tolerance capacity and act as potential bioinoculants for enhanced crop productivity in stressful environments.

Key words : Phyllosphere, Rice, Salinity, Drought, Thermal Tolerance.

Increasing crop production has presently become very challenging due to problems related to the reduction and degradation of natural resources and climate change. Abiotic stresses such as salinity, drought, temperature extremes, and pH

imbalances significantly hinder agricultural productivity. They affect the plant growth, metabolism, and yield[16]. In response to these challenges, the use of plant-associated microorganisms, particularly Plant Growth-Promoting Bacteria (PGPB), has emerged as an eco-friendly and sustainable strategy to enhance crop resilience under stress conditions[3].

Among the PGPB, pink pigmented facultative methylotrophs (PPFMs), primarily belonging to the genus *Methylobacterium*, have gained considerable attention. PPFM are strictly aerobic, rod-shaped, Gram-negative, and capable of growing on C1 compounds such as formate, formaldehyde, methanol, and methylamine[13]. These bacteria are commonly found on the phyllosphere of various plants and utilize methanol, a by-product of plant metabolism, as their sole carbon source[4]. PPFMs are known for their multiple plant-beneficial traits, including the production of phytohormones (*e.g.*, auxins and cytokinins), ACC deaminase, and exopolysaccharides, which collectively help in stress mitigation[8,11].

Although several studies have reported the plant growth-promoting effects of PPFMs under greenhouse and field conditions, there is limited understanding of their direct tolerance mechanisms under abiotic stress in vitro. Evaluating PPFMs for their intrinsic stress tolerance can help in selecting robust strains for future applications in stress-prone environments. Moreover, molecular identification using 16S rRNA gene sequencing ensures accurate classification and assessment of biodiversity among isolates.

This study aims to isolate and identify PPFM bacteria from rice plants that possess abiotic stress tolerance capacity under in vitro conditions. The findings contribute to the selection of stress-tolerant PPFM strains with potential application in sustainable agriculture under abiotic stress environments.

Isolation of PPFM isolates :

The rhizosphere soil sample and healthy rice leaves (phyllosphere) were collected from various rice cultivars (ADT 47, ADT 45, ADT 36, IR 50) grown in different locations of Cuddalore district, Tamil Nadu. The isolation of PPFM was performed using the leaf imprinting and serial dilution plate technique on Ammonium Mineral Salts (AMS) medium supplemented with 0.5% methanol (v/v) as the sole carbon source[4]. Plates were incubated at 28-30°C for 5–7 days. Based on the characteristic pink pigmentation of colonies, they were tentatively identified as PPFMs. The population of bacteria after incubation was expressed in log CFU/leaflet or g of soil sample.

Morphological and Biochemical Characterization :

Preliminary identification of isolates was performed based on morphological (colony morphology, Gram staining, and cell shape) and biochemical tests such as catalase, urease, methyl red (MR), Voges-Proskauer (VP), and starch hydrolysis[14].

In vitro Screening of Isolates for Abiotic Stress Tolerance :
Salt Stress Tolerance :

On AMS medium supplemented with

varying concentrations of NaCl (1%, 2%, and 3%), the methylotrophic isolates were examined for salt tolerance[5]. The population level of the bacteria was expressed in log CFU/ml.

Temperature Tolerance :

One ml of a 15-day-old culture suspension was added to AMS medium, and the isolates were incubated for 24 to 48 hours at 45°C, 50°C, and 55°C to assess their capacity to withstand high temperatures [7]. The population level of the bacteria was expressed in log CFU/ml.

Drought Stress Tolerance :

For testing moisture stress tolerance, the isolates were inoculated into AMS medium containing PEG 6000 at 5%, 10%, 15%, and 30% concentrations, corresponding to the osmotic potentials of -0.05 MPa, -0.15 MPa, -0.30 MPa, and -0.73 MPa, respectively[6]. Using a spectrophotometer to measure the OD value at 600 nm, growth was estimated[5].

Molecular Identification of Efficient PPFM:

Using 16S rRNA sequencing, the most efficient methylotrophic isolate was molecularly identified up to the species level. The 16S rRNA gene was amplified using universal primers 27F and 1492R after genomic DNA was extracted[2]. BLAST was performed using the NCBI GenBank database and gene sequence. To compute the evolutionary distances of organisms, the maximum composite likelihood approach was used[15]. MEGA XI software was used to create phylogenetic trees after the distance matrix was generated. Each bacterial isolate's sequencing was added to GenBank in order to obtain an accession number.

Fresh leaf and rhizosphere soil samples from rice plants were used for the isolation of methylotrophs. The phyllosphere of the rice sample (ADT 47) collected from Annamalai Nagar showed a high population of methylotrophs (6.17 log CFU per leaflet). The density of bacteria observed was low in the rhizosphere of rice (ADT 36) collected from Pennadam, Cuddalore district. Isolated PPFM colonies are shown in Fig. 1. A total of 5 isolates (three from phyllosphere and two from rhizosphere) were selected and designated

Table-1. Isolation of PPFM from different Rice growing areas of Cuddalore district, Tamil Nadu

Location	Cultivar	Habitat	Population of bacteria (Log CFU/ leaflet or g of soil)	Isolate code
Annamalai nagar	ADT 47	Phyllosphere	6.17	PPFM 1
Virudhachalam	ADT 45	Phyllosphere	5.88	PPFM 2, PPFM 3
Pennadam	ADT 36	Rhizosphere	3.49	PPFM 4
Tittakudi	IR 50	Rhizosphere	4.67	PPFM 5

Table-2. Morphological & Biochemical characterization of PPFM

Particulars	Isolated strains				
	PPFM 1	PPFM 2	PPFM 3	PPFM 4	PPFM 5
Cell shape	Rod	Rod	Rod	Rod	Rod
Colour	Dark Pink	Light pink	Pink	Pale pink	Pink
Gram reaction	Negative	Negative	Negative	Negative	Negative
Catalase test	+	-	+	+	-
Indole production test	+	+	+	+	+
Methyl red test	-	+	-	-	-
Voger – Proskauer test	-	+	-	-	-
Starch hydrolysis	-	-	-	-	-

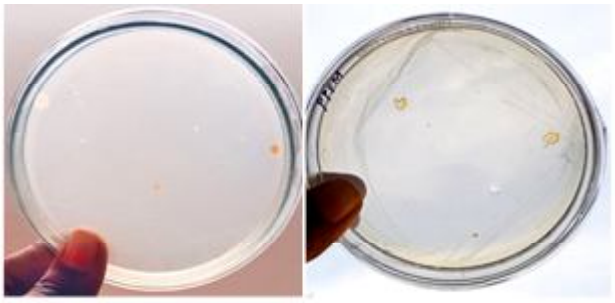

Figure 1. Colony morphology of PPFM- Pink color colony on AMS agar plate

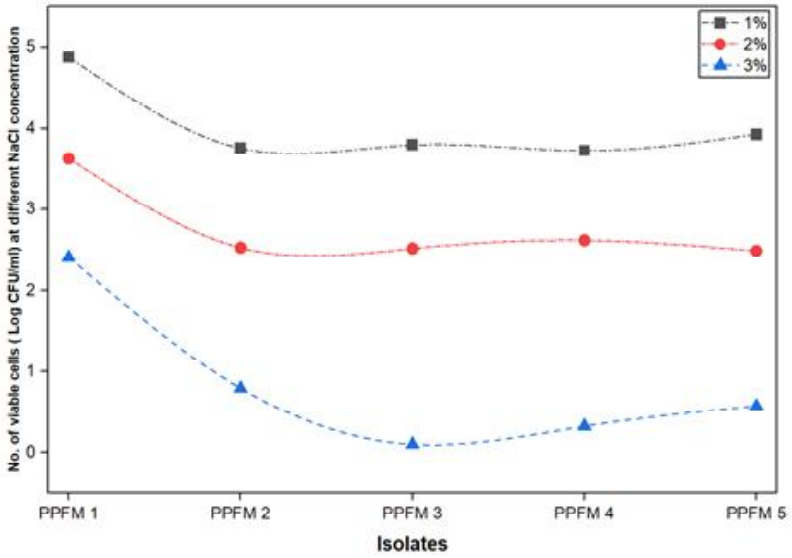

Figure 2. Population level of the bacteria (log CFU/ml) in 1-3% of salt stress

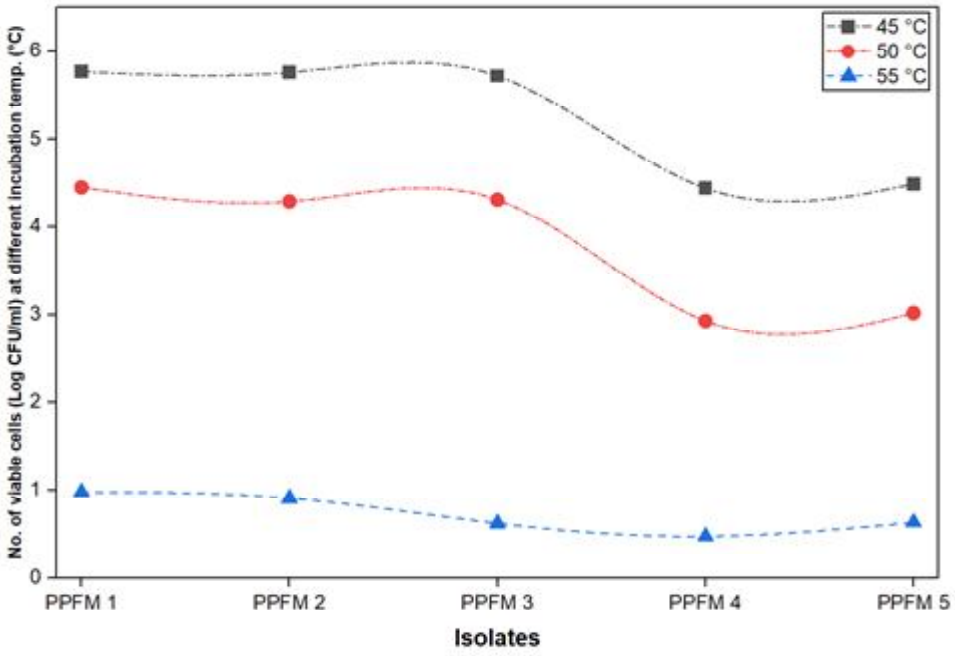

Figure 3. Population level of the bacteria (log CFU/ml) in 45- 55^{o} C thermal stress

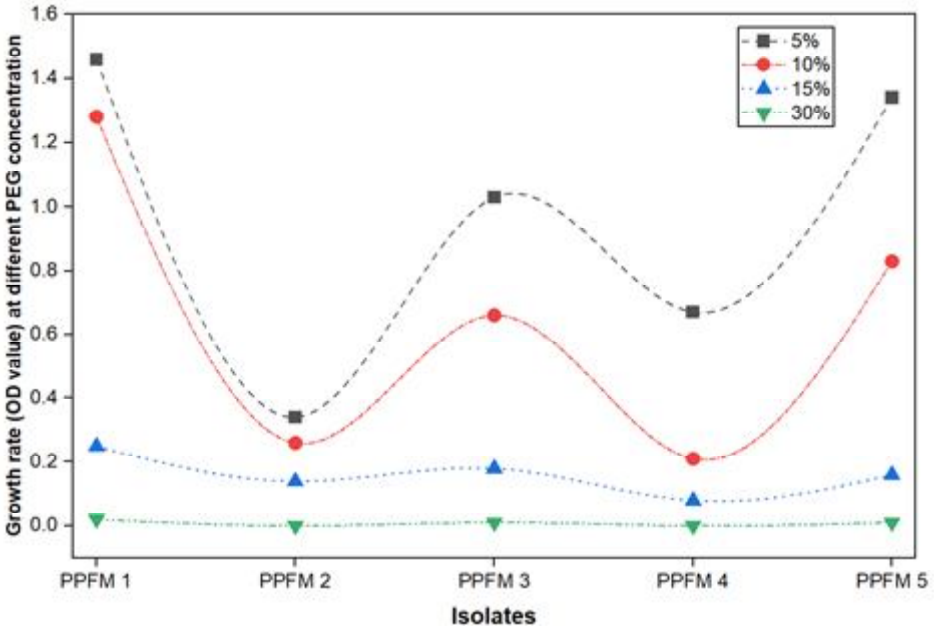

Figure 4. Growth level of bacteria (OD value at 600 nm) in moisture stress
(5- 30% of PEG 6000)

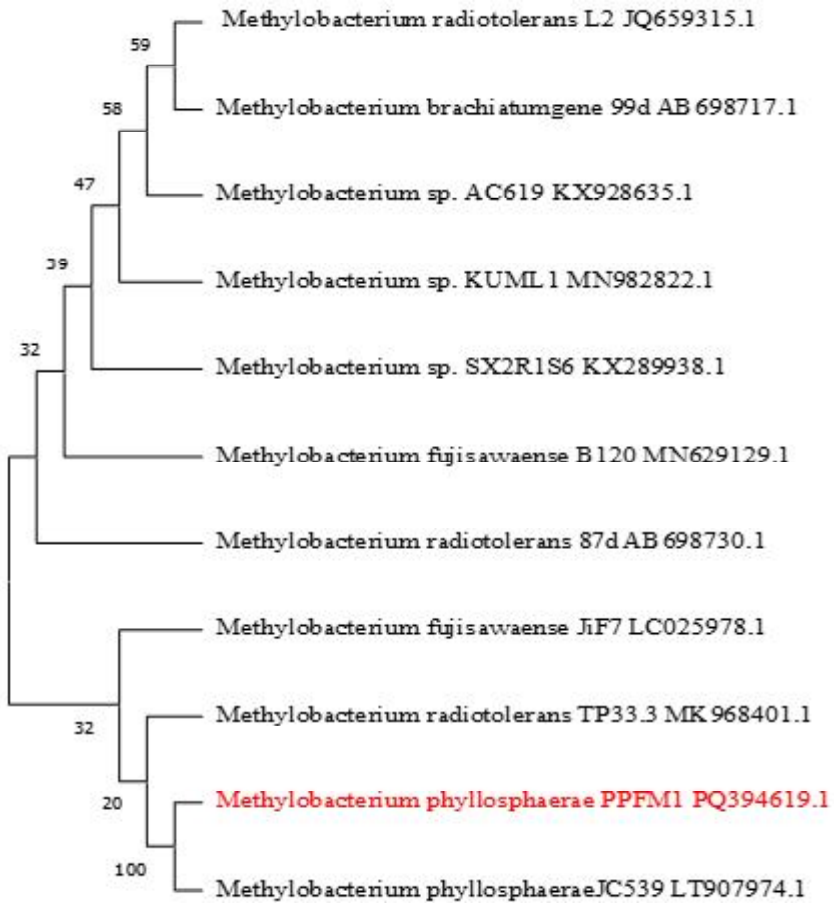

Figure 5. Phylogenetic tree of *isolate* PPFM 1 *(Methylobacterium phyllosphaerae*)

as PPFM 1 to PPFM 5 (Table-1). Further, they were used for successive subculturing and purification. Similarly, Madhaiyan *et al.* (2007) isolated a PPFM strain from the stem tissues of rice[10], and Joel *et al*. (2023) isolated it from the phyllosphere and rhizosphere of rice[5].

In morphological characterization, the methylotrophic bacterial isolates exhibited a rod shape and pale pink to dark pink colony color. Regarding Gram reaction, all isolates were found to be Gram-negative. In biochemical characterization, all the isolates were found positive towards the indole production test and negative towards the starch hydrolysis test (Table-2). Similar results were observed by Nysanth *et al*.,[12] in which five PPFM isolates from paddy were positive for oxidase, urease, catalase, and indole production[12].

The PPFM isolates were tested for salt tolerance in the respective media supplemented with 1-3% NaCl. All the isolates showed growth on an AMS medium containing

3% NaCl, ranging from 0.10 to 2.41 log CFU/ml of inoculum. The isolate PPFM 1 shows high growth even at 3% NaCl concentration when compared to other isolates (Fig. 2). The results showed similarity with the works of Ahlawat *et al.*[1] and Joel *et al.*[5], in which the bacterial isolates showed tolerance against 1-4% NaCl concentration isolated from the rhizosphere and phyllosphere[1,5].

Thermotolerance results revealed that all isolates showed growth from 45°C to 50°C, which ranges from 0.47 to 5.77 log CFU/ml of inoculum. In high temperatures (55°C) during incubation, only the isolates PPFM 1 and PPFM 2 showed more growth when compared to others (Fig. 3). Thermotolerant behavior indicates the presence of heat shock proteins or membrane stabilization mechanisms, which help survival in high temperatures[9].

In the drought-tolerant test, all isolates showed growth at 5-15% of PEG (6000) concentration, while only three isolates (PPFM 1, PPFM 3, and PPFM 5) showed minor growth at 30% of PEG (Fig. 4). In a similar study, Jorge *et al*.[6] assessed PPFM tolerance to drought conditions from 5 to 20% of PEG 6000, and the isolates with the highest O.D. values at 20% PEG concentration were considered the best plant growth promoters under water deficit conditions[6].

Out of 5 PPFM isolates, the most efficient strain, PPFM 1, was molecularly identified as *Methylobacterium phyllosphaerae* (PQ394619). The phylogenetic trees are shown in Fig. 5.

This study demonstrated the isolation of methylotrophs from rice and screening for their tolerance to abiotic stress, such as salinity, thermal stress, and drought. The results revealed isolate PPFM 1, remarkable tolerance to all mentioned stresses. It acts as the best candidate for the development of biofertilizer to improve the resilience and growth of crops in stress-prone agricultural regions. Further investigation should focus on conducting field experimental trials to confirm the efficacy of their performance under natural stress conditions.

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