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Abiotic Stress Tolerant Pink Pigmented Facultative Methylotrophs (PPFMS) Promote Plant Growth

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ABSTRACT: Pink pigmented facultative methylotrophs (PPFMs) are plant associated bacteria that utilise single carbon compounds and which have plant growth promotion potential. We have carried out the *in vitro* screening of two PPFM isolates obtained from paddy; PPFM 37 and PPFM 38 for abiotic stress tolerance and both the isolates showed tolerance to various levels of stresses *viz*., pH, salinity and water stress. Seed treatment of tomato *var.* Vellayani Vijay with these isolates improved germination and seedling growth. Seed treatment with PPFM 37 improved the growth parameters, and the dry weight recorded 28% increase over control. Hence the potential of these PPFM isolates for developing a farmer friendly commercial formulation for mitigating abiotic stress in crop plants can be explored.

Keywords: saline tolerant, water stress, PPFM, plant growth promotions, seed treatment.

INTRODUCTION

Pink pigmented facultative methylotrophs (PPFMs) form part of the plant microbiome and are often associated with the phyllosphere of crop plants. These methylotrophic bacteria possess the unique metabolic ability to utilize single carbon compounds like methane, methanol, and methylamines as their exclusive sources of carbon and energy (Anthony, 1982). PPFMs, commonly belonging to Methylobacterium species (Green and Bousfield 1982) produce pink pigmentation in artificial media. They are abundant on the above ground parts of crop plants averaging about 10^6cfu g^{-1} (Lidstrom and Chistoserdova 2002). Rajan (2003) has reported the presence of PPFMs from the phyllosphere of vegetable crops at their flowering stage. PPFMs have been detected on the leaves of brinjal, chilli, pumpkin, bitter melon, lady's finger, and tomato (Mizuno *et al*., 2013). PPFMs have garnered a lot of attention in recent years for their diverse industrial and agricultural applications.

Production of growth hormones like auxins, cytokinins, Vit B12 and enzymes are all recognized processes by which PPFMs increase plant growth. The PPFMs generate 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which captures and degrade plant-produced ACC, subsequently reducing the ethylene levels within the plant (Nysanth *et al.,* 2023). Increased ethylene levels reduce plant growth and decrease in ethylene makes it resistant to various environmental stresses. Enhancement of vegetative growth and germination, early flowering and fruit set, increased production, and drought mitigation are some of the advantageous effects of PPFMs on plants. Exploring the potential application of PPFMs to enhance plant growth and alleviate abiotic stress may result in the creation of microbial inoculants for agriculture resilient to climate change. In a preliminary study conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram twenty isolates obtained from paddy were selected based on the production of IAA, carotenoids, proline, vigour index, growth of paddy crop (Nysanth, 2018). Riyaz (2019) further evaluated these twenty isolates for their ability to mitigate drought stress in paddy and reported that PFFM 37 and PPFM 38 are promising isolates. Recognizing the significance of PPFMs in climate resilient agriculture, a study was undertaken for *in vitro* screening of these two PPFM isolates for their tolerance to abiotic stress and promotion of plant growth *in vivo*.

MATERIALS AND METHODS

Characterization of Pink Pigmented Facultative Methylotrophs (PPFMs). The *in vitro* screening of PPFMs from paddy *viz.,* PPFM 37 and PPFM 38, available in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram–was carried out for abiotic stress tolerance and plant growth promotion.

Ammonium Mineral Salt medium, supplemented with 0.5% methanol (MMS), was used as a selective medium for growing methylotrophs (Whittenbury *et al.,* 1970). The selected PPFM isolates, PPFM 37 and PPFM 38 were cultured in this media for 7 days at 28±2°C and allowed to develop the characteristic pink pigmented colonies. The colony characters, Gram

reaction, oxidase and catalase activity of the isolates were recorded.

Effect of pH on the growth of PPFM isolates. The effect of pH on the growth of the PPFM isolates was tested at acidic (5.5), neutral (7.0) and alkaline pH (8.5). Fifty mL of MMS broth was prepared in 250 mL conical flask and the pH of the medium was adjusted to 5.5, 7.0 and 8.5 with 0.1N HCl or 0.1N NaOH as per the requirement for the three treatments before autoclaving. Log phase PPFM 37 and PPFM 38 isolates were inoculated to the prepared broth and incubated at 28±2°C at 120 rpm for 7 days. The growth of the isolates on the seventh day was estimated by measuring the absorbance at 600 nm in a UV visible spectrophotometer (Shimadzu). The medium at pH 7.0 served as control.

Effect of salinity on the growth of PPFM isolates. MMS media amended with 3% and 6% sodium chloride was inoculated with PPFM 37 and PPFM 38 and incubated at 28±2°C at 120 rpm for 7 days. The optical density was measured at 600 nm. MMS medium without sodium chloride served as control.

Influence of water stress on the growth of PPFM isolates. The drought tolerance potential of the isolates was tested by estimating the growth of the isolates in AMS media amended with 5%, 10% and 20% PEG 6000 (polyethylene glycol). One mL each of log phase PPFM 37 and PPFM 38 isolates was inoculated to the prepared broth and incubated at 28±2°C at 120 rpm for 7 days in a rotary shaker. The growth of the isolates on the seventh day was measured at 600 nm. MMS medium without PEG 6000 served as control.

Production of Gibberellic Acid (GA). Gibberellic acid production by the isolates was estimated by the standard procedure (Holbrook *et al.,* 1961). For the extraction of GA from the culture broth, one mL each of the PPFM isolates was inoculated to fifty mL of AMS broth and incubated for 7–days at 28±2°C in a rotary shaker. After incubation, twenty mL of sample was centrifuged at 4000 rpm for 20 minutes at 4°C and 15 ml supernatant was collected. To this, two mL of zinc acetate solution (21.9 grams of zinc acetate + 1 mL of glacial acetic acid, made up to 100 mL with distilled water) was added. After two minutes, 2 mL of potassium ferrocyanide solution was added, and the mixture was centrifuged at 2000 rpm for 15 minutes. Following centrifugation 5 mL of 30% HCl was added to 5 mL supernatant collected in a 15 mL glass vial. It was incubated in a water bath at 20°C for 75 minutes, and the absorbance was measured at 253 nm using a UV-visible spectrophotometer (Shimadzu). The gibberellic acid in the sample was determined by referencing the standard curve.

Effect of PPFM isolates on the growth of tomato *var*. Vellayani Vijay. Seeds of tomato *var.* Vellayani Vijay procured from the Department of Vegetable Science, College of Agriculture, Vellayani, Kerala, India were used for the study. The standard plant growth promoting *Pseudomonas* strain *P. fluorescens* PN026 was used as reference culture (Nair and Anith, 2009). The experiment was set up in seedling trays, with five different treatments *viz.,* T1 - Control (Uninoculated), T2 - PN026, T3 – PPFM 37, T4 – PPFM 38 and T5- PPFM 37+ PPFM 38 and three replications per treatment.

The PPFM isolates grown in MMS broth for 7 days at 28°C (10^6 CFU mL^{-1}) was used for the seed treatment. The surface sterilized seeds were soaked in the bacterial suspension for 12 hours and shade dried before sowing. Soil and vermicompost in 1:1 ratio was filled in pro-trays. Seeds were planted in pro-trays and the seedlings maintained for 21 days and biometric observations were recorded. Ten seeds were maintained for each replication.

The germination percentage was calculated by using the formula.

$$\text{Seed germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

The dataset was subjected to a comprehensive Analysis of Variance (ANOVA) to determine the presence of statistically significant differences among the group means using GRAPES software (Gopinath *et al*., 2021)

RESULTS AND DISCUSSION

Characterization of Pink Pigmented Facultative Methylotrophs (PPFMs). The PPFM isolates, PPFM 37 and PPFM 38 developed pink colored colonies on methanol mineral salts medium (MMS) (Plate 1). The distinctive pink pigmentation of PPFMs is due to the production of carotenoids (Corpe and Basile, 1982). Both the isolates were rod shaped and Gram negative (Plate 2). All the tested organisms exhibited catalase and oxidase activity. Jourand *et al.* (2004); Madhaiyan *et al*. (2005)*;* Kumar and Lee (2009) reported positive oxidase and catalase activity of PPFMs. Thangamani (2005); Radha *et al*. (2007) also reported similar results for PPFMs.

Effect of pH on the growth of PPFM isolates. The influence of pH on the growth of PPFM 37 and PPFM 38 was assessed under three distinct pH conditions: neutral pH (7.0), acidic pH (5.5), and alkaline pH (8.5) (Table 1). The absorbance of PPFM 38 was 1.26 and PPFM 37 was 0.9487 for pH 7. The growth of the isolates was inhibited at pH 5.5 and the OD value was recorded as 0.1484 which was comparatively lower than that at neutral pH. A higher absorbance was recorded at the alkaline pH of 8.5. PPFM 37 recorded the highest OD value of 1.6572 at pH 8.5 which was considerably greater than that at neutral pH (0.9487). Jyothi Laxmi *et al.* (2012) reported that neutral to alkaline pH enhances growth and pigment development compared to acidic pH in the PPFM isolate obtained from the phyllosphere of cotton. The results of the present study are consistent with this result.

Effect of salinity on the growth of PPFM isolates. Though salinity adversely affected the growth of PPFMs *in vitro*, the isolates could tolerate salinity upto 6%. The maximum growth was recorded at 0% salinity and PPFM38 and PPFM 37 recorded 1.2573 and 0.9487 absorbance respectively. The 3% NaCl amendment in MMS medium recorded OD value of 0.5032 and 0.7811 for PPFM 37 and PPFM 38 (Table 1). However, even at 6% salinity the PPFM isolates showed growth with the absorbance value of 0.3615 for PPFM37 and 0.6656 for PPFM 38. Of the two isolates tested, PPFM

38 was found to be more tolerant to salinity as indicated by the higher absorbance. Egamberdieva *et al*. (2015) observed that *Methylobacterium mesophilicum*, isolated from the wheat rhizosphere, was capable of surviving in NaCl concentrations up to 6%.

Influence of water stress on the growth of PPFM. The growth of the cultures progressively decreased with an increase in concentration of PEG 6000 and the minimum growth was recorded at 20% PEG 6000. Though the growth of methylotrophs was restricted in the media with PEG 6000, they could grow even at 20% PEG 6000. The potential of the isolates to grow in high concentrations of PEG 6000 is an indication of their drought tolerance capacity (Table 2).

The capacity of methylotrophs to survive adverse conditions including drought was reported by several researchers (Verma *et al.,* 2014; Sivakumar *et al.,* 2017*;* Kerry *et al.,* 2018). Kumar *et al.* (2019) used various growth conditions to study abiotic stress tolerant methylotrophs. AMS media supplemented with 5–20% NaCl concentration was used for the growth of halophilic methylotrophs and 7–10% PEG amended media was used to study drought tolerant methylotrophs.

Estimation of Gibberellic Acid (GA). Recent research has shown that applying exogenous gibberellic acid (GA3) can markedly enhance seed germination, boost seed growth, improve tolerance to salinity and alleviate the inhibitory effects of salt on seedling growth (Zhou *et al.,* 2014). Both PPFM 37 and PPFM 38 produced gibberellic acid in liquid culture. GA production by PPFM 37 (16.87 ppm) was better than PPFM 38 (11.13 ppm). Savitha *et al.* (2013) reported that the GA production of PPFM isolates obtained from chilli phyllosphere and rhizosphere varied from 128.28 µg mL^{-1} to 4.77 µgmL^{-1} of culture filtrate. PPFM isolates from chilli recorded GA (30.10 to 128.28 µg mL^{-1} of culture filtrate) production in a study conducted in chilli growing areas of North Karnataka by Savitha *et al.* (2019). Virginia Joel *et al.* (2023) has reported varying levels of IAA (75 to 10.88 µg mL^{-1} of culture filtrate) and GA production (2.02 to 10.20 µg mL^{-1}) by PPFM isolates from rice. Gibberellic acid (GA) induces the secretion of hydrolytic enzymes from aleurone cells, which play a crucial role in mobilizing the endosperm storage reserves in seeds. This process is a fundamental aspect of seed germination and involves the activation of genes encoding enzymes such as α-amylase, proteases, and other hydrolases. These enzymes break down complex storage molecules like starch and proteins into simpler forms that can be transported and utilized by the developi*ng embryo for growth and energy* (Cirac *et al.,* 2004).

Effect of PPFM isolates on the growth of tomato *var.* Vellayani Vijay *in vivo*. The tomato seeds treated with the isolates germinated a day (6^{th} day) earlier than the uninoculated seeds (7^{th} day) The germination percentage was the highest in the combination treatment (81.429). This may be due to the PPFMs stimulating plant growth by producing plant hormones (Freyermuth *et al.,* 1996).

The application of PPFMs significantly increased the growth of tomato seedlings (Plate 3). Combined inoculation of PPFM 37 and PPFM 38 isolates recorded maximum root length (30.3cm). All the other treatments were found to be on par with uninoculated control with respect to root length. The application of PPFMs did not yield a statistically significant variance in shoot length. This observation suggests that, within the parameters of the study, the treatment did not exert a discernible effect on shoot elongation. The shoot weight (fresh) was maximum in the seed treatment with PPFM 37 (0.43g) followed by control and P. fluorescens PN026 treatment which were on par. In case of fresh weight of root, the best was the treatment with PPFM 37(0.58g). Control and PPFM 38 treatment recorded the lowest mean value in root fresh weight. The dry weight measurements of both plant shoots and roots exhibited analogous patterns, indicating a consistent correlation between the growth of shoot and root. The results revealed that PPFM 37 recorded maximum shoot and root dry weight (0.15g and 0.14 g). The shoot dry weight was least in the treatment with the combination while root dry weight was minimum in PPFM 38 and control. The maximum plant biomass was recorded in the treatment with PPFM 37. The fresh weight was 1.009g and the dry weight was 0.290g. The dry weight recorded 28% increase over control in the treatment with PPFM 37.

Holland (1997) reported that seed coating with PPFMs increases the germination and vigor of seeds. Ryu *et al.* (2006) documented a notable augmentation in root length among tomato and red pepper plants following seed treatment with *Methylobacterium* strains. Pushpakanth *et al*. (2021) achieved enhanced biomass of banana plantlets in greenhouse and in open environment conditions by inoculation with *Methylobacterium salsuginis* TNMB03. *Methylobacterium* inoculation has been proven to improve growth parameters of cotton (Raja and Sundaram 2006), grape vine (Jones *et al.,* 2007) and soy bean (Radha *et al.,* 2009). The biosynthesis of plant growth hormones by PPFM may aid in the accumulation of photoassimilates in different sink tissues, thereby fostering elevated levels of dry matter yield.

The present study establishes the ability of the PPFM isolates, *viz*., PPFM 37 and PPFM 38 for abiotic stress tolerance as both the isolates exhibited growth in alkaline pH of 8.5 and media amended with 6% NaCl and 20% PEG 6000. Also the portray experiment with seeds of tomato *var.* Vellayani Vijay showed that the seed treatment with PPFM isolates can promote plant growth. The methylotrophs that are in intimate association with plants can aid in plant development, increase crop productivity, and help in adaptation to a variety of abiotic conditions like drought, pH, and salinity.

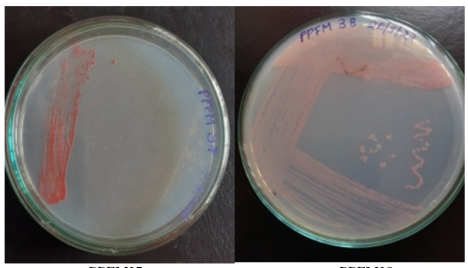

Plate 1. Pink Pigmented Facultative Methylotroph isolates in MMS agar plate.

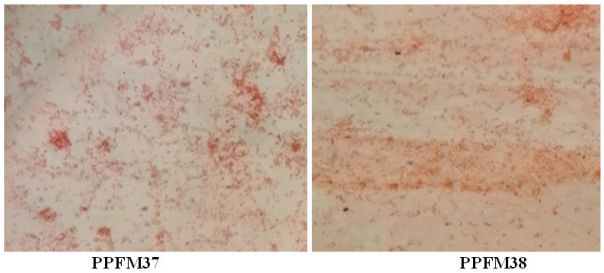

Plate 2. Gram staining of PPFM isolates.

Table 1: Effect of pH and salinity on the growth of PPFM37 and PPFM 38 (Absorbance at 600 nm).

Isolate	pH7 and 0% NaCl	pH 5.5	pH 8.5	3% NaCl	6% NaCl
PPFM37	0.9487	0.1484	1.6572	0.5032	0.3615
PPFM 38	1.2573	0.1031	0.8165	0.7811	0.6656

Table 2: Effect of PEG 6000 on the growth of PPFM37 and PPFM 38 (Absorbance at 600 nm).

Isolate	0% PEG 6000	5% PEG 6000	10% PEG 6000	20% PEG 6000
PPFM37	0.9487	0.2661	0.1085	0.1081
PPFM 38	1.2573	0.4754	0.1364	0.1121

Table 3: Effect of seed bacterization on germination and growth of tomato *var.* Vellayani Vijay.

Treatment	Seed germination	Root length of plant (cm)*	Shoot length of plant (cm)*
Control	75.714[a]	11.629[b]	6.429
PN 026	47.143[b]	18.286[b]	5.857
PPFM 37	74.286[a]	14.071[b]	8.786
PPFM 38	58.571[b]	12.571b	5.571
PPFM 37+PPFM 38	81.429[a]	30.286[a]	6.057
SEm(±)	7.076	2.898	0.824
CD(0.05)	14.451	8.369	No significant difference

Mean of 4 replications. Figure in a column followed by the same letter is not significantly different.

Table 4: Effect of seed bacterization on biomass of tomato *var.* Vellayani Vijay.

Treatment	Fresh shoot weight (g/ plant)*	Fresh root weight (g/plant)*	Dry shoot weight (g/plant)*	Dry weight of root(g/ plant)*	Total fresh biomass (g/plant)*	Total dry Biomass (g/plant)*
Control	0.284[b]	0.269[bc]	0.117[ab]	0.084[b]	0.553[b]	0.209[bc]
PN026	0.259[b]	0.364[b]	0.096[bc]	0.121[a]	0.623[b]	0.217[b]
PPFM 37	0.427[a]	0.581[a]	0.149[a]	0.141[a]	1.009[a]	0.290[a]
PPFM38	0.147[c]	0.201[c]	0.070[c]	0.076[b]	0.349[c]	0.153[c]
PPFM37+38	0.116[c]	0.364[b]	0.026[d]	0.134[a]	0.473[bc]	0.160[bc]
Sem (±)	0.038	0.053	0.013	0.012	0.071	0.021
CD (0.05)	0.111	0.154	0.038	0.035	0.204	0.061

Mean of 4 replications. Figure in a column followed by the same letter is not significantly different.

Plate 3. Effect of seed treatment with PPFMs on growth of tomato *var.* Vellayani Vijay.

CONCLUSIONS

Numerous studies have investigated the ability of various methylotrophs to withstand abiotic stress and colonize plants under extreme climate change. In the present study, both the test organisms, *viz*., PPFM 37 and PPFM 38 showed tolerance to different abiotic stresses. The ability to grow at alkaline pH of 8.5 and 6% NaCl concentration indicate the potential of the isolates to tolerate salinity. Growth in media amended with 20% PEG 6000 strongly suggest the ability of the isolates to tolerate water stress and adapt to drought conditions. The isolates also exhibited plant growth promotion ability as evident from the seed treatment experiment with the tomato *var.* Vellayani Vijay. Seed treatment with the PPFM isolates increased germination, plant length and plant biomass. PPFM 37 exhibited considerable higher values for all the growth parameters compared to control.

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FUTURE SCOPE

The preliminary work under in vitro conditions has revealed that the isolates have the potential to be developed into a farmer friendly commercial formulation for mitigating abiotic stress. However, before going into that step the potential of these isolates needs to be tested under pot and field conditions especially under abiotic stress. Because they are closely associated with crop plants, methylotrophs can promote plant growth. For improving the growth and yield of crops and soil fertility, the PGP methylotrophs may be used as bioinoculants.

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Conflict of Interest. None.

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