ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY

by

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THESIS

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DECLARATION

I, hereby declare that this thesis, entitled **"ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY"** is a record of bonafide research work done independently by **Mr. Nysanth, N. S. (2015-11-079)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

AMS	Ammonium Mineral Salts
et al.	And other co-workers
cm	Centimetre
Chl	Chlorophyll
cfu	Colony forming units
CRD	Completely Randomised Design
CD	Critical difference
DAT	Days after transplanting
°C	Degree celsius
dia	Diametre
DMSO	Dimethyl sulphoxide
Fig.	Figure
g	Gram
hrs	Hours
IAA	Indole-3- Acetic Acid
kg	Kilogram
LAI	Leaf area index
m	Metre
µg	Microgram
mg	Milligram
mL	Millilitre
mm	Millimetre
viz.	Namely
nm	Nanometre
N	Nitrogen
No.	Number

OD	Optical density
pv.	Pathovar
Ptb	Pattambi
%	Per cent
P	phosphorous
PPFM	Pink Pigmented Facultative Methylotroph
K	Potassium
Sl.	Serial
SVI	Seedling vigour index
sp.	Species
SE	Standard error
i.e.	That is
var.	Variety

INTRODUCTION

1. INTRODUCTION

The living space on the leaf surface, known as the phyllosphere, harbours a wide variety of organisms having beneficial, harmful or neutral effects on the plant. The interactions between such microorganisms and higher plants affect the physiological activities of the plant.

Pink pigmented facultative methylotrophs (PPFMs) of the genus *Methylobacterium* are commonly found in association with plants. It is hypothesized that they potentially dominate the phyllosphere bacterial population. The degree of the plant *Methylobacterium* association varies from strong, or symbiotic to loose, or epiphytic; a range that also includes the intermediate endophytic association (Lacava *et al.*, 2004). The *Methylobacterium* spp. is characterized by a distinctive pink pigmentation which is due to the presence of carotenoid pigment (Jyothilaxmi *et al.*, 2012).

PPFMs are aerobic, Gram-negative, methylotrophic rod shaped bacteria, capable of growing on a wide range of multicarbon substrates and also on single carbon compounds such as formate, formaldehyde and methanol as their sole carbon and energy source. It was assumed that a significant quantity of methanol emitted from the plant parts as a by-product of pectin metabolism during cell wall synthesis (McDonald and Fall, 1993; Nemecek- Marshall *et al.*, 1995).

Numerous species and strains of *Methylobacterium* have been isolated from plants (Knief *et al.*, 2010). PPFMs have been isolated from more than 100 species of plants ranging from liverworts and mosses to angiosperms and gymnosperms (Corpe and Basile, 1982). They are isolated on a methanol based mineral medium, Ammonium Mineral Salt (AMS) agar medium supplemented with 0.5% of methanol and cycloheximide at 100 mg L^{-1} (to inhibit fungal growth) by leaf impression method.

Many reports suggest that PPFMs can act as potential agents as plant growth promoters and also help in surviving plants from pathogenic attack (Madhayan *et al.*,

2004). They have been reported to produce plant growth regulators like zeatin and related cytokinins and auxins, which have significant effect on seed germination and seedling growth. Production of gibberellic acid (GA) by Methylobacteria has already been reported (Thangamani, 2005; Radha, 2007; Jones, 2010). Additionally, *Methylobacterium* have been reported for the production of urease enzyme (Holland and Polacco 1992), vitamin B_{12} production (Basile *et al.*, 1985), nitrogen fixation and nodule formation (Raja *et al.*, 2006), phosphate solubilization (Jones, 2010), synthesis of siderophores (Simionato *et al.*, 2006) and for the existence and prevalence of ACC deaminase enzyme (Madhaiyan *et al.*, 2006).

Considering the importance of PPFM as plant growth promoting bacteria, an attempt was made to isolate, characterize and to select the efficient PPFM strains based on *in vitro* and *in vivo* screening.

Hence the present programme has been undertaken with major thrust on the following aspects.

1. Isolation and characterization of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy.
2. *In vitro* evaluation of antagonistic efficiency of the isolates against major pathogens of paddy.
3. Evaluation of effect of isolates of PPFMs on seed germination and seedling growth and yield of paddy.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Plants produce several natural products which include carbon compounds that range in complexity from simple esters to structurally diverse compounds such as polyketides, carbohydrates, lignans, flavanoids, terpenoids, tannins, and alkaloids. Among these compounds methanol, one of the simplest organic molecules, is also a by-product of plant cell wall metabolism in rapidly growing plant organs. The methanol produced is emitted via stomatal pores of the epidermis. A likely source of methanol in the phyllosphere is the pectin demethylation in the cell walls. Pectin is structurally a hetero polysaccharide enriched with α-D-galacturonate residues, and a number of sugars such as α-L-arabinose, β-D-galactose, α-L-rhamnose in smaller amounts. Numerous galacturonate methyl esters are present in the cell wall. These compounds, present in the precursors of pectin, helps in the transport of compounds through the cell wall during cell wall expansion. Demethylation of these methyl esters by methylesterase results in the production of methanol as a by-product (Fall and Benson, 1996). Emission of methanol through stomata by the transpiration stream leads to the enrichment of plant surfaces with methanol (Nemecek-Marshall *et al.*, 1995; Huve *et al.*, 2007).

The plant phyllosphere supports a large and complex microbial community and bacteria are viewed be to the dominant microbial inhabitants of the phyllosphere. Mainly, leaves comprise a significant microbial habitat. The terrestrial leaf surface area that would possibly colonized by microbes is over 6.4 $\times$ 10^8 km^2, which support bacterial populations of about 10^{26} cells. Plant phyllosphere is an ecological niche which shelters highly abundant *Methylobacterium* species of 10^4–10^7 colony forming units (CFU) per leaflet (Mizuo *et al.*, 2012).

It has long been recognized that a number of bacteria and yeasts can not only metabolize methanol, but also utilize it as their major carbon source. These microorganisms are collectively referred as methylotrophs.

Generally, Pink pigmented facultative methylotrophs (PPFMs) of the genus *Methylobacterium* are found in association with plants. They have been considered as the dominant of phyllosphere bacterial population (Corpe and Rheem, 1989).

2.1 Methylotrophy and Methylotrophs

Methylotrophy refers to the ability of an organism to utilize single carbon compounds as the sole energy source for their growth. Several microorganisms have evolved the interesting ability to utilize single carbon (C_1) compounds (e.g. methanol or methane) or complex carbon compounds lacking carbon-carbon bonds (e.g. dimethyl ether and dimethylamine) as the sole carbon source. Microbes with this capability are known as methylotrophs (Anthony, 1986).

2.2 Pink Pigmented Facultative Methylotrophic Bacteria

Bassalik (1913) described the first *Methylobacterium* strain isolated from earth worm casts and named it as *Bacillus extorquens.* Kuono and Ozaki (1975) isolated 59 different PPFM isolates from a variety of soil and water samples. Austin *et al.* (1978) studied isolates from the phyllosphere of *Lollium perenne.* The first reported PPFM strain able to utilize methane was isolated by Patt *et al.,* (1976).

Bacteria belong to the genus *Methylobacterium* are commonly known as Pink-pigmented facultative methylotrophic bacteria. They are strict aerobic, Gram-negative, facultative methylotrophic rods. They are able to grow on C_1 compounds like methanol and methylamine and also on a variety of C_2, C_3, and C_4 compounds (Green, 1992; Trotsenko *et al.*, 2001). These common prokaryotic epiphytes are classified within the α-*Proteobacteria* and distributed in various

plants which include angiosperms, gymnosperms and even lower plants (Basile *et al.*, 1969; Austin and Goodfellow, 1979; Corpe and Basile, 1982; Corpe, 1985).

Methylobacterium are mostly known for their close association with plants and also their wide occurrence in nature (Corpe, 1985; Lidstrom and Chistoserdova, 2002). *Methylobacterium* are persistently present throughout the plant, known to inhabit the surface of leaves, stems, flowers and roots of various lower and higher plants. They have been reported as the dominant phyllosphere population from more than seventy plant species tested (Corpe, 1989).

Dileepkumar and Dube (1992) reported treatment of seeds with fluorescent Pseudomonas enhances plant growth, yield and diseases control. Similarly, PPFMs are found to have profound influence on seed germination and seedling growth. This is because of their ability to produce plant growth regulators, cytokinins and auxins (Holland and Polacco, 1992; Holland, 1997; Ivanova *et al.*, 2001; Koenig *et al.*, 2002; Omer *et al.*, 2004). They improve the growth of plants by altering agronomic traits like branching, vigor, rooting and heat or cold tolerance (Holland, 1997; Freyermuth *et al.*, 1996).

Methylobacterium spp. are gram negative rod-shaped or bacillus microorganisms. The size of the bacterium was measured to be approximately 1.0 μm long by 0.5 μm wide. Besides, the bacterial storage compound poly-β-hydroxy butyrate granules were identified in cells of *Methylobacterium spp* using PHB granule staining (Carvajal *et al.*, 2011).

2.3 Isolation of Methylotrophs

Pink Pigmented Facultative Methylotrophs (PPFMs) are ubiquitous in nature found in a variety of habitats including soil, dust, fresh water lake sediments, leaf surface and nodules.

Bassalik (1913) isolated the first *Methylobacterium* strain from earth worm casts and named it as *Bacillus extorquens*.

Patt *et al.* (1976) isolated the first PPFM strain which was able to utilize methane as the energy source.

They are found in abundance on plant leaf and stem surfaces. Phyllosphere supports different strains and species of *Methylobacterium*. Since the most common niche for synergism between *Methylobacterium* and plant is the phyllosphere, numerous species and strains of *Methylobacterium* have been isolated from the phyllosphere. Austin *et al.* (1978) studied the *Methylobacterium* strains isolated from the phyllosphere of *Lollium perenne.*

A standard procedure for the recovery of PPFMs is to make leaf impressions by pressing leaves on to the surface of plates of Ammonium Mineral Salt (AMS) agar medium supplemented with 0.5% methanol (carbon source) and cycloheximide at 100 mg l^{-1} (to inhibit fungal growth) for 1–10 min before removing the leaves. Pink and other coloured colonies will appear within 1 week and can be purified by standard procedures.

The major difficulty encountered in the PPFM isolation is that they are slow growers and if a selective medium is not used they will be overgrown rapidly by other bacteria or fungi present on plant tissues (Corpe and Rheem, 1989).

Lee *et al.* (2006) isolated three plant-growth promoting, N_2-fixing methylotrophic strains from rice cultivars (*Oryza sativa* L.) and studied the early growth promoting activities of isolated *Methylobacterium* strains in rice.

Mizuno *et al.* (2012) isolated pink pigmented facultative methylotrophs from different vegetable leaves by homogenizing the fresh leaves in ice-cooled sterilized water with Ace homogenizer at 15, 000 rpm for one minute. These homogenates were serially diluted and plated onto AMS agar medium. All kinds of vegetable leaves used for the isolation exhibited pink coloured colonies on AMS agar medium. But it has shown different cfu (colony forming units) values.

2.4 Carotenoid Pigment Production by Methylotrophs

The presence of carotenoid pigments makes them tolerant to extreme light condition and radiation. Pigmentation is common among different bacteria, especially in photosynthetic bacteria. These carotenoid pigments are involved in the harvest and transfer of light energy to the chlorophyll pigment. Hence they help to prevent the photo oxidation of chlorophyll apparatus. Pigmentation due to carotenoids helps in the easy identification of isolates as carotenoids serve as an important taxonomic markers for identification. Maximum pigment production by *Methylobacterium* grown in AMS broth was found to be at 25°C, pH of 7.5, with 0.5% of methanol (Jyothilaxmi *et al.,* 2012).

Carotenoid is an important antioxidant compound. Moreover, in photosynthetic organisms like algae and plants it act as auxiliary light harvesting pigment (Chen *et al.*, 2006; Sheehan *et al.*, 2012; Schagerl and Muller, 2006).

Godinho and Bhosle (2008) collected Gram positive bacteria from coastal sand dune vegetation, *Ipomea pes- caprae* showed a predominance of orange pigmented colonies of *Microbacterium arborescens*-AGSB. This study revealed that light induces the biosynthesis of carotenoids pigment in *Microbacterium arborescens*-AGSB which helps them to survive under stress conditions.

2.5 Beneficial Traits of Methylotrophs

Many strains of the *Methylobacterium* genus are known to promote plant growth, possibly by synthesizing plant hormones such as auxin and cytokinin and through the activity of 1-aminocyclopropane-1-carboxylate deaminase, an enzyme that lowers ethylene levels in plants.

The PPFMs are able to produce vitamin B_{12} and the addition of that vitamin to cultures of the bryophytes *Jungermannia leiantha* and *Gymnocola inflata* could stimulate plant growth and development (Basile *et al.*, 1985). While vitamin B_{12} has not been demonstrated to be essential for higher plants, there have

been reports of cobalamin-dependent enzymes from higher plants (Poston, 1977; 1978).

Many studies reported the involvement of *Methylobacterium* genus in nutrient acquisition of plants and thus association of methylotrophs with plants helps in better establishment of plants.

The methylotroph, *M. nodulans* is capable of nodulating and fixing nitrogen in symbiosis with legumes. Raja *et al.* (2006) detected the presence of functional nifH gene that is responsible for nitrogen fixation in *Methylobacterium spp.* MV10. Similarly, *M. organophilus* and *M. radiotolerans* have also been reported for nitrogen fixation and nodule formation.

Methylobacterium spp. have the ability to solubilize phosphates and thus making the unavailable phosphorus available to growing plants. They are involved in phosphate metabolism in both microorganisms and plants (Agafonova *et al.*, 2013). Jones (2010) observed the zone of solubilization by different *Methylobacterium* strains on 5th day of incubation in Pikovskaya's agar medium.

Methylobacterium spp. are able to synthesise low molecular weight compounds known as siderophores which helps in the solubilization and transport of Fe^{3+} into bacterial cells (Simionato *et al.*, 2006).

Madhaiyan *et al.* (2006) reported the existence and prevalence of ACC deaminase in *Methylobacterium*. ACC deaminase, helps to reduce the ethylene levels.

Gourion *et al.* (2006) conducted a study specifically on *Methylobacterium extorquens* and revealed the up-regulation of antioxidant-related genes and proteins when *Methylobacterium extorquens* is in association with the plant leaf.

Proline is one of the most important osmolytes that accumulate in plants during severe drought stress (Yoshiba *et al.*, 1997). It not only acts as an osmolyte for osmotic adjustment but also helps to stabilizing sub-cellular structures (e.g.

proteins and membranes). Also, involved in the scavenge of free radicals and buffering cellular redox potential. Treatment of plants with *Methylobacterium spp.* has been shown to lead to an increase in proline levels.

Meenakshi and Savalgi (2009) reported high chlorophyll content in soyabean plants, which received both seed inoculation and foliar spray of *Methylobacterium.*

2.6 Phytohormone Production by PPFMs

It is well-known that many of the soil and plant-associated bacterial groups, including Gram-negative and Gram-positive, symbiotic, and nitrogen-fixing bacteria, are able to synthesize phytohormones. Moreover, many of these bacteria have the ability to produce as well as excrete more than one phytohormone (Ryu *et al.*, 2006).

Plant hormones like cytokinins (zeatins) and auxins produced by *Methylobacterium* influenced the seed proliferation and seedling growth of several plants (Holland and Polacco, 1992, 1994; Holland, 1997; Ivanova *et al.*, 2001; Omer *et al.,* 2004). IAA synthesized by the bacteria play a significant role in the growth and development of host root system (Lee *et al.,* 2004).

Auxins are found to be essential phytohormones for normal plant growth and development, as no plant has yet been found that is unable to synthesize auxins. Among various auxins synthesized by plants, Indole-3-acetic acid (IAA) is the most important auxin. IAA producing bacteria can potentially involved in increasing the plant's auxin pool (Ryu *et al.*, 2006).

Biosynthesis of Indole-3-acetic acid (IAA) in plants occur through different pathways. Based on the intermediates formed, these pathways were classified into three; indole-3-acetamide (IAM), indole-3-pyruvic acid (IPyA), tryptamine and indole-3-acetonitrile pathway (Patten and Glick, 1996). The two most important IAA biosynthetic pathways in methylotrophic bacteria are the

IAM and the IPyA pathways (Ivanova *et al.*, 2001). IAA produced by methylotrophic bacteria via IAM and IPyA pathways has pronounced effect on plant growth.

Ivanova *et al.* (2001) first reported the production of indole acetic acid in significant amount by four different methylotrophs. Increase in plant IAA concentration was observed with the inoculation of *Methylobacterium* isolate, and also promote plant growth (Lee *et al.*, 2006).

Anitha (2010) isolated PPFMs from the phyllosphere of different crops by using leaf impression method. Eight PPFM isolates obtained were tested for their effect on seed germination and production of IAA. By using HPLC, amount of IAA produced by different isolates was estimated. A maximum of 2.32 μg mL^{-1} of IAA was produced even in the absence of tryptophan by the isolate obtained from ground nut leaf (PPFM-GN).

Based on colorimetric assay the presence of indole compounds was observed in PPFM culture supernatants (Omer *et al.*, 2004). Among the 16 isolates tested three isolates indicated the accumulation of indole compounds PPFM culture supernatants. These three isolates produced IAA in amounts ranging from 6 to 13.3 μg mL^{-1} in the presence of L-tryptophan.

Thangamani and Sundaram (2005) reported the production of IAA by *Methylobacterium* strains. The IAA produced ranged from 3.44 to 25.51 μg mL^{-1} of PPFM culture filtrate. Radha (2007) and Jones (2010) have independently documented the production of IAA by different PPFM strains ranging from 9.04 to 28.15 μg mL^{-1} and 0.14 to 25.15 μg mL^{-1} of culture filtrate, respectively.

Cytokinins are N6- substituted adenine derivatives exert potential influence on physiological processes of plants. . Root function of plants can be altered by the level of cytokinins. Involvement of plant-growth promoting rhizobacteria (PGPR) including *Azotobacter, Azospirillum, Rhizobium, Bacillus*,

and *Pseudomonas* spp. in cytokinin production in pure cultures has already been studied (Ryu *et al.*,2006).

Methylotrophic strains present on phyllosphere microbial community are not only able to produce cytokinins but also to excrete it into the culture medium (McDonald and Murrell, 1997; Ivanova *et al.*, 2000; Koenig *et al.*, 2002). The mechanism of cytokinin synthesis during association with plants requires further investigation to find whether it could be attributed to its induction in plants or to the production of cytokinins compounds by PPFMs (Holland, 1997).

Gibberellic acid (GA) production of methylobacteria has already been reported (Thangamani, 2005; Radha, 2007; Jones, 2010). These three independent studies reported that the production of Gibberellic acid (GA) by methylobacteria ranged from 28.86 to 98.26 μg mL^{-1}, 24.11 to 70.30 μg mL^{-1} and 53.2 to 273.2 μg mL^{-1} respectively. Recently, Sheela *et al.*, (2013) estimated the GA production of different *Methylobacterium* strains and the highest production was observed in PPFM 14 isolate which has produced 59.13 μg mL^{-1}.

2.7 Fungal Pathogens of Paddy

Majority of the world's population depend on rice as a food grain and is a staple food as well. Production of rice must be enhanced to meet the growing population. Biotic stresses on plants are the main problem rice cultivation. Among the various biotic stresses, diseases caused by fungal pathogens impart the most destructive effect. Among them sheath blight caused by *Rhizoctonia solani* Kuh, is a major soil borne disease causing severe economic losses to rice cultivation (Yellareddygari *et al*,. 2014).

Visible symptoms of the disease are lesion formation, lodging of plants, and grains become empty. Unfilled spikelets leads to significant reduction yield (Yellareddygari *et al.*, 2014).

Similarly, significant reduction in yield was reported when rice is infected by most devastating fungal pathogen *Magnaporthe grisea* (Hebert) Barr (anomorph: *Pyricularia oryzae* (Cooke) Sacc). The pathogen produces necrotic lesions on leaves of seedlings and on leaves, nodes, necks, and panicles of mature plants, with the latter causing the most severe yield losses (Rath and Mishra, 1975).

2.8 Bacterial Pathogens of Paddy

Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is the most damaging disease of rice. Severe infection results in poor grain development, broken rice and deterioration in chemical and nutritional composition (Ou, 1985). Hence it leads to considerable yield loss.

Symptoms of the pathogen attack is mainly seen in leaf sheaths and leaf blades. Tiny- water soaked spots are appearing edges of lower leaves at late stage of nursery. These spots become enlarged yellow lesions. Sometimes rolling and withering of leaves can be observed (Ou, 1985).

2.9 Biological Control of Diseases in Crops by Methylotrophs

Methylotrophs are known to possess antagonistic activities against several plant pathogens. Hence they can be used to protect plants from phytopathogens thereby improving the health of plants. Recently the biocontrol activity of PPFMs on soil borne phytopathogens has been recorded in addition to its promotive effect on plant growth (Madhayan *et al.,* 2004).

Seed treatment or foliar spray of *Methylobacterium* on rice induced the pathogenesis related proteins which protected the plant against sheath blight pathogen *Rhizoctonia solani* under pot culture conditions. Thus the result revealed that, application of *Methylobacterium* reduces the disease incidence in rice (Madhaiyan *et al.*, 2004).

Application of combination of four different *Methylobacterium* strains on tomato resulted in induced defence response against the plant pathogen *Ralstonia solanacearum* (Madhaiyan *et al.,* 2006).

Poorniammal *et al.* (2009) based on *in vitro* study documented the biocontrol potential of *Methylobacterium spp.* against plant pathogens, including *Sclerotium rolfsii, Fusarium udum, Fusarium oxysporum, Pythium aphanidermatum. Colletotrichum capsici, Sclerotium rolfsii, Cercospora capsici,* and *Xanthomonas campestris* with various biocontrol efficacies. Inhibition of growth of phytopathogens was measured by zone of inhibition.

Poorniammal *et al.* (2010) tested the *in vitro* biocontrol activity of four Methylobacterial isolates CO-47, MV-10, AM1 and LE-1. Among the four isolates, CO-47 significantly reduced the linear mycelial growth of *Rhizoctonia solani* to an extent of 52.2 per cent over control and the inhibition zone measured under *in vitro* conditions was 1.4 cm.

Methylotrophs isolated from mangrove sediment have been found to be potent biocontrol agent against root rot pathogen *Macrophomina phaseolina*, (Kumar *et al.*, 2015).

2.10 Mechanism of Biological Control by Methylotrophs

Studies on biocontrol potential of *Methylobacterium* has gained considerable attention. It emerges as a promising alternative to chemical control strategies. PPFMs induce several physiological changes in plants, making the plants more resistant to pathogens.

Application of *Methylobacterium extorquens* CO-47 challenge inoculated with *R. solani* to the soil induced the accumulation of peroxidase, polyphenol oxidase, phenylalanine lyase and phenols under pot culture conditions. Accumulation of enzymes suppressed *R. solani* (Madhaiyan *et al.*, 2004).

Methylobacterium spp. challenge-inoculated with *Aspergillus niger* or *Sclerotium rolfsii* in groundnut resulted in the enhancement of germination percentage and seedling vigour. It also caused the increase of phenylalanine ammonia lyase (PAL), β-1,3- glucanase, and peroxidase (PO) activities. A pot culture study has been conducted in which groundnut seeds were treated with *Methylobacterium* spp. Later, the groundnut plants challenge-inoculated with *A. niger or S. rolfsii* through foliar spray on 30th day resulted in a constant increase in activities of enzymes PO, PAL and β-1,3-glucanase from 24 to 72 hours, followed by decreased activity was noted. Also detected the presence of five isozymes of polyphenol oxidase and PO in *Methylobacterium* treated plants challenged with *A. niger* or *S. rolfsii* (Madhaiyan *et al.*, 2006).

Methylobacterium spp. synthesize pathological factors, such as siderophores (Simionato *et al.,* 2006). Bacterial siderophores are low-molecular-weight compounds with high Fe^{3+} chelating affinity responsible for the solubilization and transport of this element into bacterial cells. There are two types of siderophores; hydroxamate-type and catecholate-types. *Methylobacterium spp.* have no ability to produce catechol-type siderophores, but are capable to produce hydroxamate-type siderophores (Lacava *et al.*, 2008). The siderophores produced by microorganisms can inhibit the growth of plant pathogens by limiting the availability of Fe^{3+}.

The antagonistic effect on the fungal pathogens tested may also be attributed to the salicylic acid production capability of PPFM isolates as already proved in *Methylobacterium oryzae* CBMB20 challenge inoculated with *Pseudomonas syringae* pv. *tomato* in tomato plants compared to either control or *M. oryzae* treated tomato plants in both growth chamber and green-house conditions (Indiragandhi *et al.,* 2008).

Poorniammal *et al.* (2009) studied the production of volatile antibiotics by *Methylobacterium* and the effect on the mycelial growth of *F. oxysporum* and *F. udum.* There was a linear relationship, greater the amount of volatile compounds

produced by the *Methylobacterium*, less the radial growth of mycelium and more the inhibition percentage. Of four *Methylobacterium* strains tested, CO-47 produced more volatile compounds, which significantly reduced the mycelial growth of *F. oxysporum* compared to other isolates.

2.11 Impact of PPFM on Drought Stress Alleviation in Plants

Increased incidences of abiotic and biotic stresses impacting productivity in major crops are being witnessed all over the world. Among these drought stress is the major threat to principal crops. The problem of drought is increasing continuously with reduction in production of crops (Qayyum and Malik, 1988). Plants' tolerance to drought stress needs to be improved in order to allow growth of crops that satisfy food demands under limited water resource availability.

Bacterial inoculants that provide cross protection against both biotic and abiotic stress would be highly preferable for environmentally sustainable agricultural systems (Van Loon *et al.*, 1998).

Madhaiyan *et al.* (2006) reported the presence of ACC deaminase in *Methylobacterium fujisawaense* and its lowering of ethylene levels and promotion of root elongation in canola seedlings under gnotobiotic conditions.

Hayat *et al.* (2010) reported that exogenous application of PPFM produces some benefit in alleviating the adverse effects of drought stress and also improves germination, growth, development, quality and yield of crop plants.

Gawad *et al.* (2015) investigated the effect of PPFM bacteria on the antioxidant enzymes, growth and yield of snap bean plants. Results revealed that application of plants with PPFM individually or combined with methanol changed the level of antioxidant enzymes including polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD). This study proved the positive effect of PPFM the growth and yield of snap bean plants.

Sivakumar *et al.* (2017) assessed the impact of PPFMs and plant growth regulators on alleviating the drought stress effects in tomato. The study indicated that the PPFMs and PGRs could be effectively improving drought tolerance capacity of tomato crop under drought. Among the three different concentrations of PPFM (PPFM (1%), PPFM (2%) and PPFM (3%)) used, PPFM (2%) was found to superior in improving relative water content, photosynthetic rate, SPAD value and proline content of tomato plant.

2.12 Methylotrophs Associated with Crops

Several species of methylotrophic bacteria are found in association with terrestrial and aquatic plants, colonizing roots, leaf surfaces, and growing buds (Trotsenko *et al.*, 2001 ; Lidstrom and histoserdova, 2002).

Inoculation of *Methylobacterium* and methanol spray significantly increased plant height and dry matter production in cotton than uninoculated control (Madhaiyan, 2003).

Radhika *et al.* (2008) recorded highest maize cob yield in plants sprayed with PPFMs.

Pattanashetti *et al.* (2012) conducted a pot culture experiment to study the effect of selected methylotrophs on growth and yield of *Coleus forskohlii*. Results suggested that plant height, chlorophyll content, shoot biomass, leaf area, stem girth and tuber yield increased due to PPFMs treatment. Among the various isolates tested PPFM50 was selected as the best. Inoculation of this isolate increased the tuber yield to 216.10% against uninoculated control and 136.07% against reference strain.

Madhaiyan *et al.* (2006) reported the presence of ACC deaminase, enzyme which reduces the level of ethylene, in *Methylobacterium fujisawaense*. Lower levels of ethylene promote the root elongation of canola seedlings under gnotobiotic conditions.

A two-year field experiment revealed inoculation of PPFM alone achieved the highest significant increases in the number of leaves per plant, average leaf area, haulm fresh weight, leaf chlorophyll, pod number and yield per plant. In addition, it improved the quality of pods by increasing the amino acids, protein, total sugars and ascorbic acid content (Gawad *et al.*, 2015).

True seeds of sugarcane treated with PPFMs resulted in higher germination percent and rate of germination compared to the control. A combination of PPFM treatment (seed imbibitions, soil application and phyllosphere spray) increased plant height, specific leaf area, number of internodes and cane yield (Madhaiyan *et al.,* 2005).

Rice, the most important cereal crop in the world, belongs to the family Poaceae. It is the most widely consumed staple food for over half of the world's population. For the last several years, the production potential of rice has remained the same. It is mainly due to constrains like increased population, declined area under cultivation, threats from climate change, evolution of newer biotypes of pest and diseases, causing serious challenges to rice producers. There are several reports on the beneficial effects of PPFMs on paddy. They promote the growth of paddy in a number of ways.

Lee *et al.* (2006) observed the effect of three plant-growth promoting, N2 fixing methylotrophic strains *Methylobacterium spp.* CBMB20, *Enterobacter* sp. CBMB30, *Burkholderia spp.* CBMB40, on the early growth of rice. These three methylotrophic strains significantly improved seed germination, seedling vigour index (SVI) and biomass of rice seedlings.

Eight *Methylobacterium* isolates were tested for their effect on seed germination. The isolates, PPFM-SOY (isolated from soybean leaf) and GN (isolated from groundnut leaf) increased the germination percentage of heat-treated seeds of soybean, maize and paddy. When the heated seeds of soybean was treated with PPFM-SOY, 14.28 per cent increase in germination was obtained compared to untreated heated seeds. Same level of increase in

germination was observed on treatment with PPFM-GN. When normal seeds were treated with PPFM-SOY and PPFM-GN, 23.21 and 7.14 per cent increase in germination was observed respectively. Treatment of heated maize seeds with PPFM-SOY and PPFM-GN resulted in an increase of 27.50% and 30.0% over control respectively. For paddy seeds also 13.88% and 11.11% increase over control was recorded on treatment with PPFM-SOY and PPFM-GN respectively (Anitha, 2010).

Madhaiyan *et al.* (2004) observed higher photosynthetic activity in rice cultivar Co-47 that received *Methylobacterium* and attributed the effect due to enhancement of chlorophyll concentration, maleic acid content and increased number of stomata.

Application of PPFMs, either foliar spray or irrigation with aqueous methanol, ethanol or both was found to improve the plant growth and pod quality. They also contribute to the aroma in strawberry (Yavarpanah *et al.*, 2015).

2.13 Taxonomic Characterization of PPFMs

2.13.1 Cultural Characteristics

Studies on the growth characteristics revealed that *Methylobacterium spp.* are fairly slow growers. It takes almost 2 to3 days at either 25°C or 30°C in order to reach the size of visible colonies. Often it takes more than 7 days for colonies to reach their maximum size (Garrity *et al.*, 2005). Investigations regarding the doubling time of *Methylobacterium* have been done in three different culture media. Results revealed that its doubling time is approximately one hour and also reported the slow growth of *Methylobacterium* either in minimal or enriched medium (Guzman and Pamaong, 2008).

2.13.2 Colonial and Microscopic Morphology

All the *Methylobacterium* isolates identified so far had exhibited similar colonial morphology. It was entire, convex, butyrous consistency. Growth on

enrichment media Glycerol Peptone Agar (GPA) had shown raised opaque colonies. Pigmentation of the colony ranges from pink to orange in color. The colonial morphology and pigmentation serve as an important taxonomic marker for the identification of *Methylobacterium* grown in Ammonium Mineral Salt agar media (AMS) (Lee, 2010).

2.13.3 Morphological Characteristics of *Methylobacterium* Strains

Methylobacterium spp. are rod shaped or bacillus microorganisms of about average size 1.0 μm long and 0.5 μm wide (Carvajal *et al.*, 2011). Although many of these rods occur in singly, sometimes remains in rosettes (Patt *et al.*, 1976).

They are often branched or pleomorphic, especially in older stationary phase cultures. They exhibit polar growth or budding morphology. Also, all strains show motility, possess single polar, sub-polar or lateral flagellum (Heumann, 1962).

In addition, poly-β-hydroxybutyrate granules are observed in *Methylobacterium* cells and are identified using PHB granule staining (Carvajal *et al.*, 2006; Jang and Lee, 2008; Kumar and Lee, 2009; Lee, 2010; Carvajal *et al.*, 2011)

Most of the studies have shown that *Methylobacterium* are gram negative, however some reports observed to be gram variable (Green and Bousifield, 1982).

When grown on glycerol peptone agar medium they exhibit entire, convex, butyrous consistency and pigmentation of the colony may differ. Size of the colonies varies from 1 to 3 mm in diameter, while the pigmentation of the colony ranges from pale pink to orange red (Lee, 2010).

2.13.4 Biochemical Characteristics of *Methylobacterium* Strains

All the reported isolates were aerobic which produces both catalase and oxidase (Bellin and Spain 1976). Therefore, all the *Methylobacterium spp.* are

catalase and oxidase positive (Green, 1992).They are chemoorganotrophs and facultative methylotrophs capable of growing on a variety of C1 compounds such as methanol, formaldehyde, methylamine and also, complex carbon compounds lacking carbon-carbon bonds (e.g. dimethyl ether and dimethylamine).

Lipolytic activity is very weak in all the *Methylobacterium* strains reported so far. Most of the strains are not able to hydrolyse starch, gelatin, cellulose, lecithin or DNA. They do not produce enzymes such as β-galactosidase, L-ornithine decarboxylase, L-lysine decarboxylase and L-arginine dihydrolase. The methyl red and Voges-Proskauer tests are negative, although some strains reduce nitrate to nitrite. Thangamani (2005) reported that methylotrophs are positive for urease test and indole production.

2.13.5 Carbon Utilization Tests for *Methylobacterium* Strains

Based on the pattern of compounds they utilize as carbon and energy source, Lidstrom (1992) differentiated *Methylobacterium* species, concluded that they are capable of growing on single compounds as sole source of carbon and energy and can also grow on wide range of multicarbon substrates making them facultatively methylotrophic. The compounds that were used by more than 95 per cent of *Methylobacterium* strains include, methylamine, trimethylamine, acetate, citrate, L-glutamate, D-glucose, D-xylose, fructose and betaine and carbon source utilization pattern by 12 known species of the genus *Methylobacterium* revealed that none of the strains appear to use any of the disaccharides or sugar alcohols examined but most of *Methylobacterium* strains used glycerol, malonate, succinate, fumarate, α-ketoglutarate, D,L-lactate, D,L-malate, acetate, pyruvate, propylene glycol, ethanol, methanol and formate as carbon and energy sources (Green and Bousifield, 1982).

2.13.6 Molecular Taxonomy of PPFMs

Molecular techniques are most authoritative tools for the identification of an organism. These techniques provide significant information of a species.

Jones *et al.* (2007) cloned and sequenced the amplicon of the 16S rDNA from a mineral phosphate solubilizing methylobaterium for the first time and later confirmed that the native isolate to be a close relative of *Methylobacterium fujisawaense.*

Balchander *et al.* (2008) studied six diversified groups of Pink-Pigmented Facultative Methylotrophs (PPFMs) in phyllosphere of cotton, maize and sunflower through differential carbon substrate utilization profile and Random Amplified Polymorphic DNA (RAPD).

Marx et al. (2012) detected complete genome sequences of six strains of *Methylobacterium.* All the strains showed considerable variation in chromosome size and plasmid content. Each strain showed several conserved gene clusters known to be involved in methylotrophy.

Kwak *et al.* (2014) isolated plant growth-promoting methylotroph, *Methylobacterium oryzae* CBMB20T and analyzed the complete genome sequence. The genome consists of a 6.29-Mb chromosome and four plasmids, designated as pMOC1 to pMOC4. Among the 6,274 coding sequences in the chromosome most of the genes are associated with central metabolism, while all other essential genes are associated with assimilation and dissimilation of methanol that are either located in methylotrophy islands or dispersed.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The experiment on the "Isolation and characterization of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy" was carried out during the period from 2015-2017 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram. The details of the materials used and methods followed in the present study are presented in this chapter.

3.1 ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY.

3.1.1 Collection of Leaf Samples

The leaf samples of paddy were collected from different agro climatic conditions of Kerala. The samples were brought to the laboratory in sterile polythene bags and stored at 4^0C.

3.1.2 Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs).

Ammonium Mineral Salts (AMS) medium (Whittenburry *et al.*, 1970) is a selective medium for isolation of methylotrophs. The AMS medium was sterilized by autoclaving at 121°C for 15 min and cooled to 45°C. Filter sterilized vitamin solution (Colby and Zatman, 1973) along with 0.5 per cent (v/v) methanol was added after sterilization and before pouring media on to petriplates. The pH of the medium was adjusted to pH 7.0.

On the solidified AMS agar medium upper and lower surface of leaf samples were placed separately, in such a way as to make impression of it. Then the leaves were lifted away and plates were incubated at 30^0C for 7 days (Corpe, 1985). Based

on characteristic pink pigmentation of colonies they were tentatively identified as PPFMs.

3.1.3 Purification of Pink Pigmented Facultative Methylotrophs (PPFMs).

PPFMs obtained by leaf imprint technique were purified by the streak plate method and well isolated colonies on the plates were preserved on Peptone Glycerol agar (enrichment medium) slants at 4°C in a refrigerator for further use.

3.2 ESTIMATION OF INDOLE ACETIC ACID AND CAROTENOID PIGMENT PRODUCTION BY THE DIFFERENT ISOLATES OBTAINED.

3.2.1 Estimation of Indole Acetic Acid Production by the Different Isolates Obtained.

Indole Acetic Acid was estimated as per the procedure described by Gordon and Weber (1951).

100 ml of AMS broth supplemented with 0.05 per cent methanol and cycloheximide was prepared in 250 ml flasks. To this medium, 0.1 per cent tryptophan was added. Using sterile technique, the medium was inoculated with one ml of PPFM inoculum (10^7 cfu/ ml). Flasks were kept for incubation at 30^0C for 7 days. After incubation, culture was centrifuged at 10000 rpm for 10 minutes. To the 10 ml of culture supernatant 2 ml of the Salkowski reagent was added. Incubated at room temperature for 25 minutes and then read at OD_{530}.

Using the standard curve for IAA, the amount of IAA was calculated.

3.2.2 Estimation of Carotenoid Pigment Production by the Different Isolates Obtained

Carotenoid pigment was estimated as per the procedure described by Harborne (1976).

The PPFM culture was grown in AMS medium for 7 days in a shaker at 250 rpm at 30^0 C. 100 ml of this broth was centrifuged at 9000 rpm for 10 minutes. After centrifugation wet pellet weight was taken. The pellet was dissolved in 80% acetone and incubated the tubes at 60^0C for 15 minutes. After centrifugation at 9000 rpm for 10 minutes, supernatant absorbance was measured at 470nm, 663 and 647 nm using spectrophotometer. Using the standard graph of carotenoid pigment, the amount of carotenoids pigment was calculated using the given formula,

$$C\ (\mu g/ml) = (1000A_{470} - 1.82A_{663} - 85.02A_{647}) /198$$

3.3 EVALUATION OF ANTAGONISTIC EFFICIENCY OF THE ISOLATES FOR DISEASE SUPPRESSION

The efficiency of the PPFM isolates to suppress major pathogens of paddy such as *Rhizoctonia solani, Xanthomonas oryzae* pv. *oryzae* and *Pyricularia oryzae* was tested by dual culture technique (Dennis and Webster, 1971). The fungal pathogens *i.e; Rhizoctonia solani* and *Pyricularia oryzae* were obtained from the culture collection of Department of Agricultural Microbiology, College of Agriculture, Vellayani and the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* was obtained from the culture collection of RARS, Mancompu. Mixed media of potato dextrose agar and peptone glycerol agar medium in the proportion of 1:1 was used for testing the antagonistic activity of PPFM isolates against fungal pathogens. For testing the antagonistic activity of PPFM isolates against bacterial pathogen, mixed medium of 1:1 proportion of potato sucrose agar medium and peptone glycerol medium was used in the investigation.

3.3.1 Antagonistic Activity of PPFM Isolates Against Fungal Pathogens *Rhizoctonia solani* and *Pyricularia oryzae*

Dual culture technique for fungal pathogens *Rhizoctonia solani* and *Pyricularia oryzae* was done on mixed medium (1:1 proportion of potato dextrose

agar and peptone glycerol agar medium). Mycelial disc (8mm) of fungal pathogens placed at centre of petriplates. Two streaks using fresh suspension of bacterial isolates made on opposite sides of plate. Plates were incubated at 30^0 C for 7 days. After incubation, size of inhibition zone (mm) was measured.

3.3.2 Antagonistic Activity of PPFM Isolates Against Bacterial Pathogen *Xanthomonas oryzae* pv. *oryzae*

Dual culture technique for bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* was done on mixed medium (1:1 proportion of potato sucrose agar medium and peptone glycerol medium). Bacterial pathogen was spreaded on the plate using spread plate method. Sterile disc was dipped in 7 days old liquid culture (10^7 cfu/ ml) of PPFM and placed at the centre of the plates aseptically. Plates were incubated at 30^0 C for 7 days. After incubation, size of inhibition zone (mm) was measured.

3.4 EFFECT OF ISOLATES OF PPFM ON PADDY SEED GERMINATION

Seeds of variety Jyothi were surface sterilized before treatment with the bacterial suspension. The seeds were first washed with sterile distilled water twice and then treated with 70% ethanol for 1 min. This was followed by treatment with 2% sodium hypochlorite solution for 30 seconds. Finally, the treated seeds were thoroughly rinsed for more than five times with sterile distilled water. Surface sterilized seeds were soaked overnight in 1 per cent of 7 days old liquid culture (10^7 cfu/ ml) of the respective isolates. After decanting the liquid culture, the dried seeds were placed on filter paper in a petri dish. Plates were incubated at 30^0 C for 72 hrs. The untreated seeds were kept as control.

3.4.1 Observations

3.4.1.1 *Days Taken for Germination*

Observations on seed germination were monitored daily. Recorded the time taken for germination for each treatment.

3.4.1.2 Percentage Seed Germination

The germination percent was calculated after 72 hrs. After taking the number of germinated seeds, percentage seed germination was calculated using the formula,

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

3.4.2 Effect of Isolates of PPFM on Paddy Seedling Growth.

To calculate the effect of PPFM inoculation on seedling vigor index of paddy, the seeds were surface sterilized with 70% alcohol and 0.1% mercuric chloride, which was followed by a series of washings with sterile distilled water. Surface sterilized seeds were soaked overnight in 1 per cent of 7 days old liquid culture ((10^7 cfu/ ml) of the respective isolates and sown in plastic pots filled with wetland soil. The untreated seeds were taken as control (Plate 1).

3.4.2.1 Observations

3.4.2.1.1 Shoot Length

After 14 days the shoot length was measured from base of the stem to the tip of the longest leaf and expressed in cm.

Plate 1. Effect of PPFM isolates on seedling growth of paddy

3.4.2.1.2 Root Length

After 14 days the root length was measured from base of the stem to the tip of the root and expressed in cm.

3.4.2.1.3 Seedling Vigour Index (Baki and Anderson, 1973)

Seedling vigour index was calculated using the formula,

Seedling Vigour Index = Germination Percent x (Shoot length + Root length)

3.4.2.1.4 Fresh Weight of Shoot

The fresh weight of Shoot (mg) was taken in an electronic single pan balance immediately after uprooting the plants.

3.4.2.1.5 Fresh Weight of Root

The fresh weight of root (mg) was taken in an electronic single pan balance immediately after uprooting the plants.

3.4.2.1.6 Dry Weight of Shoot

The dry weight of shoot (mg) was taken after drying the samples to a constant weight at 60 °C in a drying oven.

3.4.2.1.7 Dry Weight of Root

The dry weight of root (mg) was taken after drying the samples to a constant weight at 60 °C in a drying oven.

3.4.2.1.8 Root Shoot Ratio

After taking the dry weight of shoot and root (mg), Root Shoot ratio was calculated using the formula,

$$\text{Root Shoot Ratio} = \frac{\text{Dry weight of root (g)}}{\text{Dry weight of shoot (g)}} \times 100$$

3.5 EFFECT OF PPFM ISOLATES ON GROWTH OF PADDY

A pot culture experiment was conducted to assess the effect of PPFM isolates on biometric characters, yield and yield attributes, proline, chlorophyll content and per cent cell membrane integrity of paddy in the Department of Agricultural Microbiology, Vellayani, Thiruvananthapuram (Plate 2).

3.5.1 Preparation of Pots

The wetland soil belonged to the textural class of sandy clay and the taxonomical order Oxisol and recorded a pH of 5.41 and EC of 0.4 dSm^{-1} collected from paddy fields of College of Agriculture, Vellayani was filled into earthen pots of 30 cm diameter at the rate of 10.5 kg per pot.

3.5.2 Fertilizer Application

Fertilizers were applied as per the recommended dose of 70:35:35 kg NPK per hectare. N in the form of urea, P in the form of rajphos and K in the form of muriate of potash were applied to soil.

Plate 2. Field view of pot culture experiment

3.5.3 Preparation of PPFM Inoculum

The PPFM bioinoculant was prepared by inoculating 72 h old log phase culture in AMS broth (Whittenburry *et al.*, 1970). The flasks were kept in a temperature controlled shaker at 28±2^{0}C for 7 days.

3.5.4 Nursery Bed Preparation

The rice (var. Jyothi (Ptb-39)) seedlings were raised in the nursery bed of size 1m × 1m. The recommended package of practices were followed in nursery.

3.5.5 Details of Pot Culture Experiment

Location	: College of Agriculture, Vellayani.
Crop	: Rice
Variety	: Jyothi (Ptb-39)
Design	: Complete Randomized Design
Treatments	: 48
Replications	: 2
Number of plants/replication	: 2

Treatments

$T_1 - T_{46}$: Forty six PPFM isolates obtained from different locations of Kerala.

T_{47} : Reference culture (The isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as reference culture).

T_{48} : Uninoculated control

3.5.6 Seedling Dip Method

The 15 days old healthy seedlings were selected for transplanting. The seedlings were uprooted from nursery bed and dipped in 2 percent solution (10^5 cfu/ ml) of PPFM culture for 30 min before transplanting. After root dip two seedlings were transplanted per earthen pot containing 10.5 kg wetland soil.

3.5.7 Spraying of PPFMs on Leaves

The PPFM cultures were grown for 7 days and prepared 1 percent solution (10^5 cfu/ ml) and sprayed with a hand sprayer at the rate of 25 ml/plant on the leaves in the morning to have uniform wetting as described by Holland and Polacco (1994) at 15 and 30 days after transplanting (DAT).

3.5.8 Observations

3.5.8.1 Biometric Characters

The different biometric observations recorded from each treatment are presented below:

3.5.8.1.1 Plant Height (cm)

The height of the plant was measured from the base to the growing tip of the shoot in cm at 30, 60 and 90 DAT.

3.5.8.1.2 Number of Tillers Hill^{-1}

Recorded as the total number of productive and non productive tillers at 30, 60 and 90 DAT.

3.5.8.1.3 Leaf Area Index

Leaf Area Index was calculated at 50 % flowering stage. Leaf Area Index was computed by the following formula developed by Watson (1947).

$$\text{Leaf Area Index} = \frac{\text{Leaf area plant}^{-1}\ (\text{cm})^2}{\text{Land area occupied by the plant}\ (\text{cm})^2} \times 100$$

3.5.8.2 Yield and Yield Attributes

3.5.8.2.1 Number of Panicles Hill^{-1}

Total number of panicles from each hill was counted and expressed as number of panicles hill^{-1}.

3.5.8.2.2 Panicle Length

Length of main axis of five randomly selected panicles of observational plants was measured from base to tip and the average expressed in 'cm'.

3.5.8.2.3 Number of Grains Panicle^{-1}

The entire spikelets including filled and unfilled grains were counted from five panicles and the mean number of spikelets panicle^{-1} was worked out.

3.5.8.2.4 Number of Filled Grains Panicle^{-1}

Recorded from five randomly selected panicle and the mean number of filled grains panicle^{-1} was calculated.

3.5.8.2.5 Sterility Percentage

The number of filled and unfilled grains panicle^{-1} was obtained from ten randomly selected panicle separately and sterility percentage was worked out using the following formula:

$$\text{Sterility percentage} = \frac{\text{Number of unfilled grains per panicle}}{\text{Number of total grains per panicle}} \times 100$$

3.5.8.2.6 Thousand Grain Weight

One thousand grains were counted from cleaned and dried produce from the observational plants and the weight of the grains was recorded in 'g'.

3.5.8.2.7 Grain Yield Hill^{-1}

Plants were harvested from the pot, threshed, cleaned, dried to 14 per cent moisture, weighed and expressed the grain yield hill^{-1} in g hill^{-1}.

3.5.8.2.8 Straw Yield Hill^{-1}

The straw obtained from each hill was dried to constant weight under sun, weighed and the straw yield hill^{-1} expressed in g hill^{-1}.

3.5.8.2.9 Dry Matter Production Hill^{-1}

The plants from each pot were harvested at 107 DAT and kept in brown paper bags having holes to enable air-drying and then oven dried at 70^{0}C to achieve

constant weight. Afterwards, the plant dry weight was recorded and expressed as dry matter per hill^{-1}.

3.5.8.3 Chlorophyll Content

Chlorophyll content was estimated by DMSO (Dimethyl sulphoxide) method. A weighed quantity of sample (0.5g) was taken and cut into small bits. These bits were put in test tubes and incubated overnight at room temperature, after pouring 10ml DMSO : 80% acetone mixture (1:1v/v). The coloured solution was decanted into a measuring cylinder and made upto 25ml with the DMSO-acetone mixture. The absorbance was measured and readings were taken at 663, 645, 480 and 510nm using spectrophotometer. The chlorophyll content was expressed as mg g^{-1}.

Chl a = $(12.7 \times A_{663} - 2.69 \times A_{645}) \times V/1000 \times 1/\text{fresh weight}$

Chl b = $(22.9 \times A_{645} - 4.68 \times A_{663}) \times V/1000 \times 1/\text{fresh weight}$

Total Chl (a+b) = $(8.02 \times A_{663} + 20.2 \times A_{645})\ V/1000 \times 1/\text{fresh weight}$

3.5.8.4 Proline Content

Proline content was estimated as per the procedure described by Bates *et al.*, (1973). A known quantity (0.5g) of mid-leaf portion was homogenized with 10ml of 3% aqueous sulphosalicylic acid and centrifuged at 3000 rpm for 15 minutes. 2ml of the supernatant was taken and mixed with an equal quantity of glacial acetic acid and acid ninhydrin. The contents were allowed to react at 100^{0}C for one hour in water bath. The reaction was terminated by keeping it in ice bath for 10 min. The reaction mixture was mixed with 4ml toluene using vortex mixture for 15 – 20 seconds. The chromophore containing toluene was aspirated from aqueous phase, warmed to room temperature and the optical density was read at 520nm with toluene as blank. A standard curve was drawn using concentration verses absorbance.

The concentration of proline was determined from graph and expressed as

$$\mu \text{ moles/g tissue} = \{[(\mu g \text{ proline / ml}) \times \text{ml toluene}] / 115.5\} \times (5 / \text{g sample}),$$

where 115.5 is the molecular weight of proline.

3.5.8.5 Cell Membrane Stability Index

Cell membrane stability was estimated as per the procedure described by Blum and Ebercon (1981). Samples collected from both control and PPFM inoculated plants were washed three times in deionised water to remove electrolytes adhered on the surface. Samples were kept in a capped vial (20ml) containing 10ml of deionised water and incubated in the dark for 24 hours at room temperature. The conductance was measured with a conductivity meter. After the first measurement the vials were autoclaved for 15 minutes to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for both control and PPFM treated plants. Cell membrane stability index was calculated by using following formula and expressed as per cent.

$$CMS (\%) = [1-(T_1/T_2)/1-(C_1/C_2)] \times 100$$

Where, T and C refer to the treated and control samples respectively. The subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

3.6 CHARACTERIZATION OF ISOLATES OF PPFM

3.6.1 Morphological Characterization of Isolates of PPFM

The following morphological tests viz. cell shape, gram reaction and motility were carried out to characterize the tentatively identified PPFM isolates.

3.6.1.1 Cell Shape

The purified cultures at log phase were observed microscopically for the cell morphological characters (Becking, 1974).

3.6.1.2 Cell Motility

The 72 h old cells were observed microscopically using cavity slide for their motility.

3.6.1.3 Gram Reaction

Gram staining was carried out as per modified Hucker's method (Rangaswami and Bagyaraj, 1993) and observed under the microscope.

3.6.2 Biochemical Characterization of Isolates of PPFM

Biochemical characterization of selected bacterial isolates was done by performing various biochemical tests and carbohydrate utilization tests by using readymade Himedia© kits (HiCarbo™, Part A, Band C, Hi25™ Enterobacteriaceae). Colour change observed on the biochemical amended media of the kit after spot inoculating culture suspensions of selected isolates followed by incubation for 72 h indicated the reaction with respect to different biochemicals or carbohydrates as positive or negative. Various biochemical tests performed were Citrate utilization, Lysine utilization, Ornithine utilization, Urease, Phenylalanine Deamination, H_2S production, Nitrate reduction, Catalase, Arginine Lyase, Malonate utilization and Voges Proskauer etc. Different carbohydrate utilization tests performed were Glucose, Adonitol, Lactose, Sorbitol, ONPG, Sucrose, Mannitol, Trehalose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Melibiose, L-arabinose, Mannose, Inulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbitol, Adonitol, Arabitol, Erithritol, Alpha Methyl D-glucoside, Rhamnose, Cellobiose, Melezitose, Alpha Methyl D-mannoside, Xylitol, Esculin hydrolysis, D- arabinose and Sorbose

methylamine, tartarate, betaine, sebacic acid, dimethylamine, ethanol, methanol etc. The results of biochemical tests were utilized to arrive at a tentative genus level identification of isolates.

3.6.3 Molecular Characterization of Isolates of PPFM

Molecular characterization of bacterial isolates were done by 16S rRNA cataloging using universal primers at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

3.6.3.1 Genomic DNA Isolation

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

3.6.3.2 Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis.

3.6.3.3 PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile 16S rRNA

95 °C - 5.00 min

95 °C - 30 sec
60 °C - 40 sec
} 35 cycles
72 °C - 60 sec

72 °C - 7.00 min

4 °C - ∞

3.6.3.4 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the

bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.3.5 ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.6.3.6 Sequencing using BigDye Terminator v3.1

Sequencing reaction was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4

minutes.

3.6.3.7 Post Sequencing PCR Clean up

1. Master mix I- 10µl milli Q and 2 µl 125mM EDTA per reaction.
2. master mix II-2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol.
3. 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.
4. 52 µl of master mix II was added to each reaction.
5. Contents were mixed by inverting and incubated at room temperature for 30 minutes
6. Spun at 14,000 rpm for 30 minutes
7. Decanted the supernatant and added 100 µl of 70% ethanol
8. Spun at 14,000 rpm for 20 minutes.
9. Decanted the supernatant and repeated 70% ethanol wash
10. Decanted the supernatant and air dried the pellet.

The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.6.3.8 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained

sequences were carried out using Geneious Pro v5.6 (Drummond *et al.*, 2012). Sequence similarity was checked using Basic Local Alignment Tool (BLAST).

3.7 STATISTICAL ANALYSIS

The data obtained from the studies conducted under laboratory and field conditions were subjected to analysis of variance (ANOVA) after appropriate transformations wherever needed. In the case where the effects were found to be significant, critical difference values were calculated for each observations using 't' values at 5 per cent level of significance . Then the significance of treatments was compared with critical difference values. All the data were analysed in 'OPSTAT' developed by CCS Haryana Agricultural University.

RESULTS

4. RESULTS

The present study on "Isolation and characterization of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy" was conducted during the period from 2015-2017 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results based on statistically analyzed data pertaining to the experiment conducted during the course of investigation are presented below.

4.1 ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY.

4.1.1 Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs) Associated with Paddy.

The pink pigmented facultative methylobacteria (PPFM) were isolated from the phyllosphere of paddy, collected from different locations of Kerala by leaf imprint method using Ammonium Mineral Salt (AMS) agar media supplemented with 0.5% methanol and cycloheximide.

Forty six isolates were obtained from different locations and allotted code numbers for each of the isolate (Table 1). They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on AMS agar media with methanol as sole source of carbon and energy (Plate 3, 4 and 5). The isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as reference culture.

4.2 ESTIMATION OF INDOLE ACETIC ACID AND CAROTENOID PIGMENT PRODUCTION BY THE DIFFERENT ISOLATES OBTAINED.

4.2.1 Estimation of Indole Acetic Acid Production by the Different Isolates Obtained.

Table 1. Different locations of leaf sample collection

Sl. No	Isolate code No.	Place
1	PPFM1	College of Agriculture, Vellayani.
2	PPFM2	College of Agriculture, Vellayani.
3	PPFM3	College of Agriculture, Vellayani.
4	PPFM4	College of Agriculture, Vellayani.
5	PPFM5	College of Agriculture, Vellayani.
6	PPFM6	College of Agriculture, Vellayani.
7	PPFM7	College of Agriculture, Vellayani.
8	PPFM8	College of Agriculture, Vellayani.
9	PPFM9	College of Agriculture, Vellayani.
10	PPFM10	College of Agriculture, Vellayani.
11	PPFM11	Ookode,Balaramapuram.
12	PPFM12	Ookode,Balaramapuram
13	PPFM13	Ookode,Balaramapuram
14	PPFM14	Ookode,Balaramapuram
15	PPFM15	Ookode,Balaramapuram
16	PPFM16	Ookode,Balaramapuram
17	PPFM17	Ookode,Balaramapuram
18	PPFM18	Ookode,Balaramapuram
19	PPFM19	Chitoor, Palakkad.
20	PPFM20	Chitoor, Palakkad.
21	PPFM21	Chitoor, Palakkad.
22	PPFM22	Chitoor, Palakkad.
23	PPFM23	Chitoor, Palakkad.
24	PPFM24	Attappadi, Palakkad.
25	PPFM25	Pattambi, palakkad.
26	PPFM26	Pattambi, palakkad.
27	PPFM27	Pattambi, palakkad.
28	PPFM28	Pattambi, palakkad.
29	PPFM29	Karamana, Thiruvananthapuram
30	PPFM30	Karamana, Thiruvananthapuram
31	PPFM31	Karamana, Thiruvananthapuram
32	PPFM32	Karamana, Thiruvananthapuram
33	PPFM33	Karamana, Thiruvananthapuram
34	PPFM34	Mancompu, Alappuzha.
35	PPFM35	Mancompu, Alappuzha.
36	PPFM36	Mancompu, Alappuzha.
37	PPFM37	Mancompu, Alappuzha.
38	PPFM38	Mancompu, Alappuzha.
39	PPFM39	Mancompu, Alappuzha.
40	PPFM40	Punchakari, Thiruvananthapuram

41	PPFM41	Punchakari, Thiruvananthapuram
42	PPFM42	Punchakari, Thiruvananthapuram
43	PPFM43	Punchakari, Thiruvananthapuram
44	PPFM44	Punchakari, Thiruvananthapuram
45	PPFM45	Punchakari, Thiruvananthapuram
46	PPFM46	Punchakari, Thiruvananthapuram
47	PPFM47 (Reference strain)	TNAU

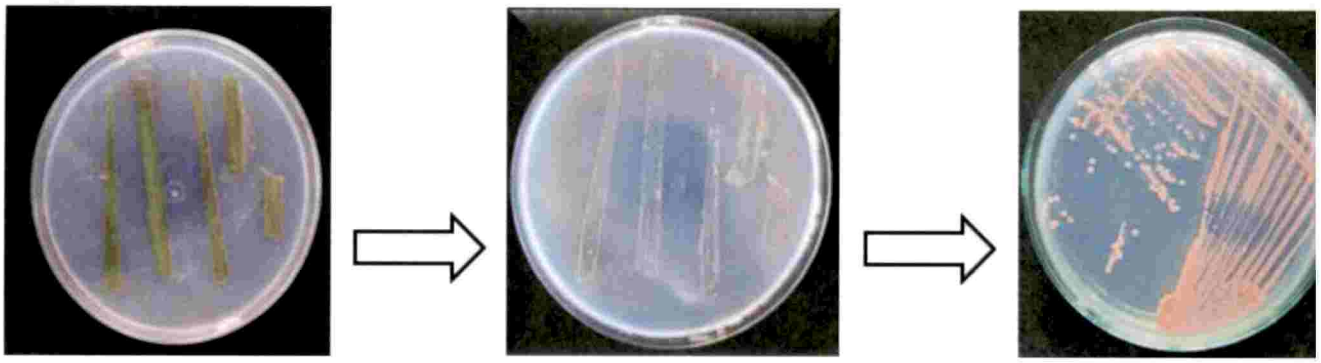
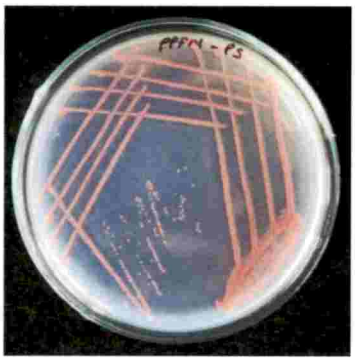


Plate 3. Isolation of Pink Pigmented Facultative Methylotrophs (PPFMs) by leaf impressi method on AMS agar medium

(A) (B)

Plate 4. (A) Liquid culture of PPFM isolates (B) Maintenance of PPFM culture in slants

PPFM1



PPFM6

PPFM8

PPFM11

PPFM12

PPFM16

PPFM19

PPFM22

PPFM25

PPFM35

PPFM38

PPFM42

Plate 5. Different PPFM isolates obtained by leaf impression method

Indole- Acetic Acid production by the different isolates of PPFMs showed wide variations ranging from 9.27 to 68.65 µg $mL^{-1}$of culture filtrate. The details are presented in Table 2.

Maximum IAA production of 68.65µg $mL^{-1}$of culture was recorded by PPFM35 followed by PPFM37 (67.81 µg mL^{-1}) which were found to be statistically on par with each other. The lowest IAA production was recorded by PPFM1 (9.27 µg mL^{-1}). The reference culture produced 57.39 µg mL^{-1} of IAA.

4.2.2 Estimation of Carotenoid Pigment Production by the Different Isolates Obtained.

All the isolates which were found to produce carotenoid pigment in varied amount ranging from 0.07 to 1.74 µg mL^{-1}. The results are presented in Table 2.

Of the total isolates, the highest carotenoid production was recorded in PPFM16 (1.74 µg mL^{-1}) followed by PPFM17 (1.57 µg mL^{-1}) which were statistically on par with each other. Significantly lowest pigment production was recorded by PPFM1 (0.07 µg mL^{-1}) and PPFM18 (0.07 µg mL^{-1}). The reference culture recorded a total carotenoid production of 0.64 µg mL^{-1}.

4.3 *IN VITRO* EVALUATION OF ANTAGONISTIC EFFICIENCY OF THE ISOLATES AGAINST MAJOR PATHOGENS OF PADDY.

The antagonistic activities of all the PPFM isolates were assessed against important fungal pathogens of paddy namely, *Rhizoctonia solani, Pyricularia oryzae* and one bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* following dual culture method and the results are furnished below:

4.3.1 Antagonistic Activity of PPFM Isolates Against *Rhizoctonia solani*

Six out of forty seven isolates tested, inhibited *Rhizoctonia solani.* PPFM10 exhibited the maximum zone of inhibition (ZOI) of 12.72 mm diameter which is significantly superior to all other isolates. It was followed by the isolate

Table 2. Indole-acetic acid (IAA) and carotenoid pigment production by the PPFM isolates

Sl. No.	Isolate code No.	IAA ($\mu g\ mL^{-1}$)*	Total carotenoids ($\mu g\ mL^{-1}$)*
1	PPFM1	9.27	0.07
2	PPFM2	24.62	0.30
3	PPFM3	45.19	0.23
4	PPFM4	39.65	0.23
5	PPFM5	30.96	0.52
6	PPFM6	16.08	0.73
7	PPFM7	19.54	0.62
8	PPFM8	26.23	0.39
9	PPFM9	27.42	0.39
10	PPFM10	49.31	0.41
11	PPFM11	27.38	0.62
12	PPFM12	30.00	1.05
13	PPFM13	40.38	0.79
14	PPFM14	47.62	0.46
15	PPFM15	39.89	0.59
16	PPFM16	34.38	1.74
17	PPFM17	40.27	1.57
18	PPFM18	28.19	0.07
19	PPFM19	34.73	0.88
20	PPFM20	29.31	0.45
21	PPFM21	23.11	1.07
22	PPFM22	32.08	0.61
23	PPFM23	39.92	0.33
24	PPFM24	22.96	0.28
25	PPFM25	19.23	0.57
26	PPFM26	24.96	0.21
27	PPFM27	29.08	0.22
28	PPFM28	33.89	0.55
29	PPFM29	63.15	0.26
30	PPFM30	25.23	0.21
31	PPFM31	32.81	0.15
32	PPFM32	54.96	0.19
33	PPFM33	51.00	1.29
34	PPFM34	41.19	0.79
35	PPFM35	68.65	0.56

36	PPFM36	18.23	0.69
37	PPFM37	67.81	0.96
38	PPFM38	51.57	0.18
39	PPFM39	39.81	0.70
40	PPFM40	35.73	0.42
41	PPFM41	22.96	0.63
42	PPFM42	50.27	0.67
43	PPFM43	12.92	0.84
44	PPFM44	18.58	0.72
45	PPFM45	33.12	0.91
46	PPFM46	40.23	0.92
47	PPFM47 (Reference strain)	57.39	0.64
	CD (0.05)	1.526	0.266
	SEm (±)	0.54	0.09

*Mean of 2 independent replications

PPFM47 (reference strain) with a zone of inhibition of 9.07 mm which was statistically on par with PPFM3 (8.00 mm), PPFM7 (7.87 mm), PPFM36 (7.25 mm) and PPFM44 (7.22 mm) isolates (Plate 6) (Table 3).

4.3.2 Antagonistic Activity of PPFM Isolates Against *Xanthomonas oryzae* pv. *Oryzae*

Four out of forty seven isolates tested inhibited, *Xanthomonas oryzae* pv. *oryzae* (Table 4). PPFM5 produced the maximum ZOI of 9.80 mm diameter which was statistically on par with ZOI produced by PPFM10 (9.30 mm) and PPFM1 (8.97 mm). These three isolates were found to be significantly superior to all other isolates. The lowest ZOI of 6.90 mm was observed with PPFM28 (Plate 7).

4.3.3 Antagonistic Activity of PPFM Isolates Against *Pyricularia oryzae*

Four isolates (PPFM9, PPFM11, PPFM14 and PPFM24) showed antagonistic activity against *Pyricularia oryzae* (Table 5). PPFM24 exhibited the maximum zone of inhibition of 10.00 mm which was statistically on par with other isolates PPFM9, PPFM11 and PPFM14 which produced ZOI of 9.75, 9.25 and 6.75 mm respectively (Plate 8).

4.4 EFFECT OF ISOLATES OF PPFM ON PADDY SEED GERMINATION AND SEEDLING GROWTH.

4.4.1 Days Taken for Germination

Both treated and uninoculated control seeds germinated on the second day of seed treatment.

4.4.2 Percentage Seed Germination (%)

The data on the effect of PPFM isolates on seed germination are cited in Table 6.

Table 3. Antagonistic activity of PPFM isolates against *Rhizoctonia solani*

Sl. No.	Isolate code No.	ZOI (mm)*
1	PPFM 3	8.00[b]
2	PPFM 7	7.87[b]
3	PPFM 10	12.72[a]
4	PPFM 36	7.25 [b]
5	PPFM 44	7.22[b]
6	PPFM 47 (Reference strain)	9.07[b]
	CD (0.05)	2.529
	SEm (±)	0.84

*Mean of 4 replications

Table 4. Antagonistic activity of PPFM isolates against *Xanthomonas oryzae* pv. *oryzae*

Sl. No.	Isolate code No.	ZOI (mm)*
1	PPFM 1	8.97[a]
2	PPFM 5	9.80[a]
3	PPFM 10	9.30[a]
4	PPFM 28	6.90[b]
	CD (0.05)	1.176
	SEm (±)	0.35

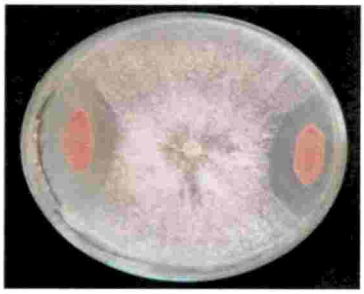
*Mean of 4 replications

Table 5. Antagonistic activity of PPFM isolates against *Pyricularia oryzae*

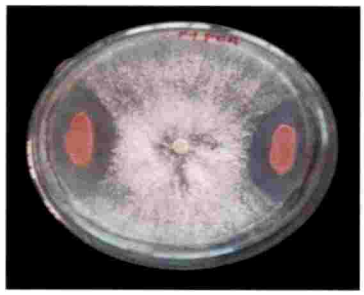
Sl. No.	Isolate code No.	ZOI (mm)*
1	PPFM9	9.75[a]
2	PPFM11	9.25[a]
3	PPFM14	6.75[b]
4	PPFM24	10.00[a]
	CD (0.05)	2.190
	SEm (±)	0.71

*Mean of 4 replications

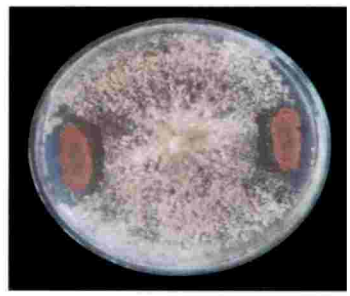
Growth of *Rhizoctonia solani* on potato dextrose agar medium



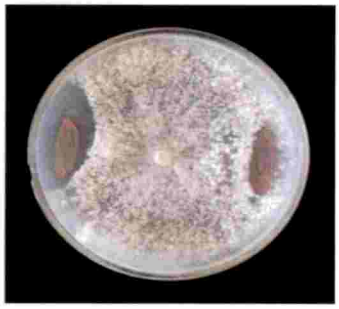
PPFM10



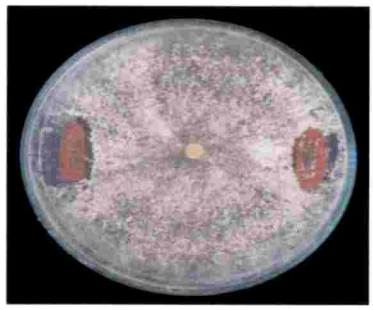
PPFM47



PPFM3



PPFM7

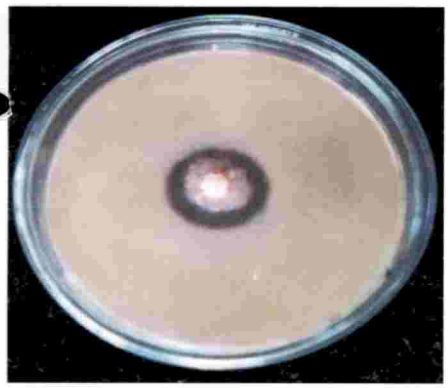


PPFM36

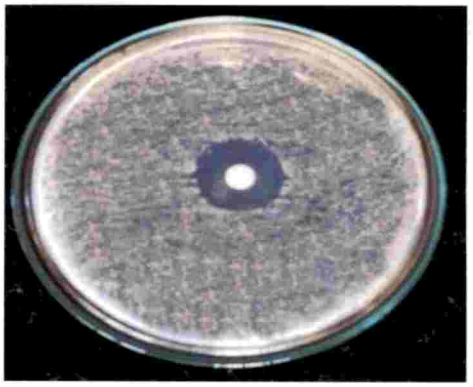
PPFM44

Plate 6. Antagonistic activity of PPFM isolates against *Rhizoctonia solani*

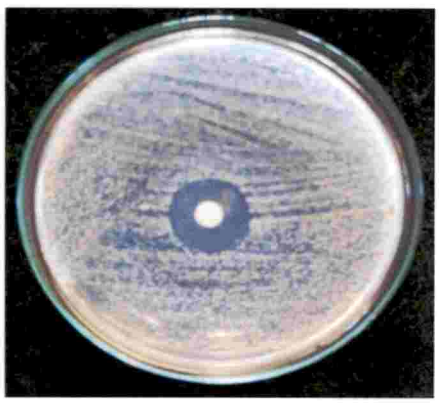
Growth of *Xanthomonas oryzae* pv. *oryzae* on potato sucrose agar medium



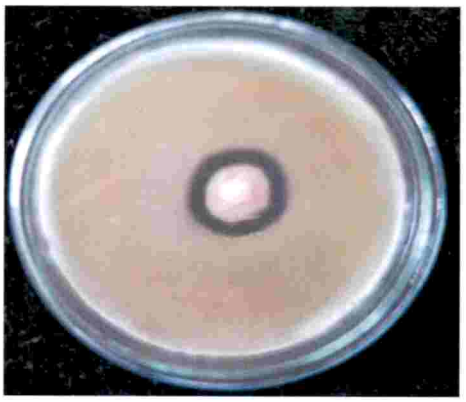
PPFM5



PPFM10



PPFM28

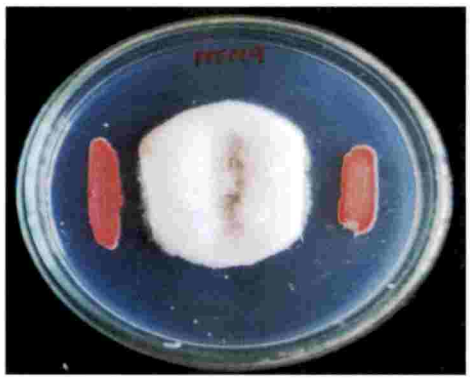


PPFM1

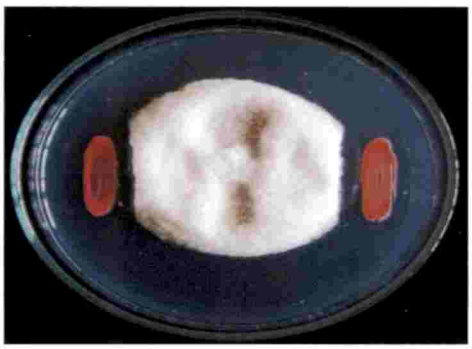
Plate 7. Antagonistic activity of PPFM isolates against *Xanthomonas oryzae* pv. *oryzae*

Growth of *Pyriculria oryzae* on potato dextrose agar medium

PPFM24



PPFM9



PPFM11

PPFM14

Plate 8. Antagonistic activity of PPFM isolates against *Pyriculria oryzae*

Table 6. Effect of PPFM isolates on paddy seed germination

Sl. No.	Isolate code No.	Seed germination (%)
1	PPFM1	93.00
2	PPFM2	93.00
3	PPFM3	94.00
4	PPFM4	87.67
5	PPFM5	84.33
6	PPFM6	89.67
7	PPFM7	89.67
8	PPFM8	96.00
9	PPFM9	95.00
10	PPFM10	96.00
11	PPFM11	98.00
12	PPFM12	93.00
13	PPFM13	92.33
14	PPFM14	90.67
15	PPFM15	87.67
16	PPFM16	94.00
17	PPFM17	75.33
18	PPFM18	90.67
19	PPFM19	95.00
20	PPFM20	79.33
21	PPFM21	86.33
22	PPFM22	96.00
23	PPFM23	79.67
24	PPFM24	95.00
25	PPFM25	93.00
26	PPFM26	94.00
27	PPFM27	98.00
28	PPFM28	84.67
29	PPFM29	97.00
30	PPFM30	98.00
31	PPFM31	95.00
32	PPFM32	93.00
33	PPFM33	91.00
34	PPFM34	94.00
35	PPFM35	100.00
36	PPFM36	92.00

37	PPFM37	92.00
38	PPFM38	93.00
39	PPFM39	77.67
40	PPFM40	97.67
41	PPFM41	90.67
42	PPFM42	96.00
43	PPFM43	86.00
44	PPFM44	90.00
45	PPFM45	84.67
46	PPFM46	88.00
47	PPFM47 (Reference strain)	94.00
48	Control	86.00
	CD (0.05)	6.922
	SEm (±)	2.46

Even though both treated seeds and uninoculated control germinated on the second day of seed treatment, the germination percentage of inoculated seeds showed a significant increase compared to uninoculated control. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35. However this was statistically on par with the treatments PPFM11 (98.00), PPFM27 (98.00), PPFM30 (98.00), PPFM40 (97.67), PPFM29 (97.00), PPFM8 (96.00), PPFM10 (96.00), PPFM22 (96.00), PPFM42 (96.00),PPFM9 (95.00), PPFM19 (95.00), PPFM24 (95.00), PPFM31 (95.00), PPFM3 (94.00), PPFM16 (94.00), PPFM26 (94.00), PPFM34 (94.00) and reference strain, PPFM47 (94.00). These treatments were found to be significantly superior to the uninoculated control which recorded a germination percentage of 86 per cent.

4.4.3 Shoot Length (cm)

Significant increase in seedling shoot length was observed when seeds were treated with PPFM isolates. Inoculation with PPFM30 recorded the maximum shoot length of 26.38 cm followed by PPFM8 (25.72 cm), PPFM19 (25.25 cm), PPFM3 (25.02 cm), PPFM11 (25.00 cm), PPFM2 (24.62 cm), PPFM14 (24.40 cm), PPFM15 (23.80 cm) and PPFM6 (23.60 cm).These treatments were found to be significantly superior to the uninoculated control which recorded a shoot length of 17.84 cm (Table 7). The reference culture treated seedlings recorded a shoot length of 23.03 cm.

4.4.4 Root Length (cm)

Observation on root length of PPFM isolates inoculated seeds, after 14 days of sowing showed a significant increase compared to uninoculated control. Maximum root length of 24.20 cm was obtained in seeds treated with PPFM22. This was statistically on par with PPFM16 (24.10 cm), PPFM12 (22.60 cm) and PPFM11 (22.40 cm) (Table 7). All these treatments were significantly superior to the uninoculated control and reference strain which recorded a root length of 17.50 and 18.90 cm respectively (Plate 9).

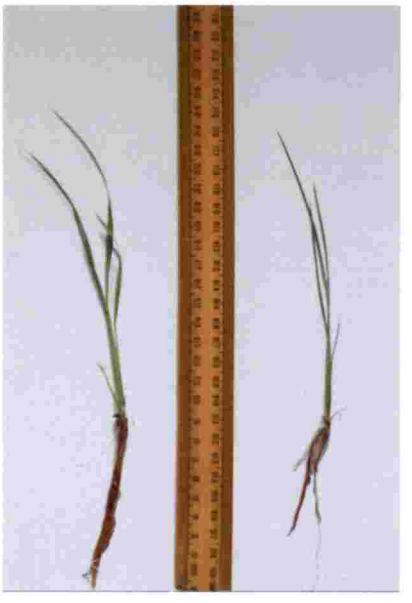
PPFM22 Control

PPFM30 Control

PPFM8 Control

PPFM19 Control

PPFM3 Control



PPFM11 Control

Plate 9. Effect of PPFM isolates on shoot and root growth of paddy seedlings

4.4.5 Seedling Vigour Index

Paddy seeds treated with PPFM22 recorded the highest seedling vigour index of 4756.35 (Table 7) and this was significantly superior compared to the control which recorded a vigour index of 3037.91. However this treatment was statistically on par with PPFM11 (4646.83), PPFM16 (4465.37), PPFM9 (4515.58) and PPFM8 (4402.82). The reference culture recorded a seedling vigour index of 3943.45.

4.4.6 Fresh Weight of Shoot (g)

The data indicated that seedling shoot fresh weight was significantly influenced by PPFM treatments (Table 8). Inoculation with PPFM8 recorded the highest shoot fresh weight of 0.67 g and was statistically on par with other treatments viz, PPFM6, PPFM9, PPFM 14, PPFM16, PPFM15, PPFM20 and PPFM19 which recorded a shoot fresh weight of 0.65, 0.63, 0.62, 0.60, 0.60, 0.60 and 0.58 g respectively. The control recorded a shoot fresh weight of 0.57 g. The shoot fresh weight of reference culture was 0.54 g.

4.4.7 Fresh Weight of Root (g)

A perusal of the data showed that treatment of paddy seeds with PPFM had significant influence on root fresh weight of seedlings (Table 8). Among all the treatments, PPFM16 recorded the highest root fresh weight of 0.35 g. This was statistically on par with the treatments PPFM35 (0.34 g), PPFM45 (0.34 g), PPFM26 (0.33 g), PPFM19 (0.30 g) and PPFM33 (0.30 g). All these treatments were statistically superior to control which recorded a root fresh weight of 0.24 g. The root fresh weight of reference culture was 0.28 g.

4.4.8 Dry Weight of Shoot (g)

Significantly higher dry weight of shoot was observed in seedlings when seeds were treated with PPFM isolates. PPFM42 treated seeds recorded a maximum shoot dry weight of 0.37 g. This was followed by PPFM24, PPFM6,

Table 7. Effect of PPFM isolates on shoot length, root length and seedling vigour index of paddy seedlings

Sl. No.	Isolate code No.	Shoot length (cm)/ seedling*	Root length (cm)/ seedling*	Seedling Vigour Index
1	PPFM1	19.58	17.93	3,489.06
2	PPFM2	24.62	19.70	4,121.48
3	PPFM3	25.02	17.90	4,039.46
4	PPFM4	19.75	22.80	4,155.06
5	PPFM5	23.50	18.86	3,716.88
6	PPFM6	23.60	20.13	3,939.10
7	PPFM7	20.03	17.70	3,380.53
8	PPFM8	25.72	20.17	4,402.82
9	PPFM9	21.95	20.87	4,515.58
10	PPFM10	19.77	19.67	3,784.00
11	PPFM11	25.00	22.40	4,646..83
12	PPFM12	21.25	22.60	4,073.94
13	PPFM13	21.25	18.57	3,656.82
14	PPFM14	24.40	18.40	3,885.80
15	PPFM15	23.80	21.50	3,963.71
16	PPFM16	23.38	24.10	4,465.37
17	PPFM17	20.12	17.97	2,849.70
18	PPFM18	23.17	17.70	3,707.40
19	PPFM19	25.25	18.80	4,181.97
20	PPFM20	23.61	20.03	3,465.24
21	PPFM21	24.82	21.30	3,978.15
22	PPFM22	25.38	24.20	4,756.36
23	PPFM23	24.07	17.93	3,345.03
24	PPFM24	25.38	20.03	4,313.27
25	PPFM25	22.60	19.23	3,889.83
26	PPFM26	24.75	18.70	4,090.04
27	PPFM27	26.25	19.07	3,719.78
28	PPFM28	23.50	18.97	3,570.58
29	PPFM29	20.55	19.73	3,904.60
30	PPFM30	26.38	17.57	4,285.96
31	PPFM31	25.85	14.20	3,813.18
32	PPFM32	23.68	18.27	3,900.67
33	PPFM33	21.37	18.23	3,604.07
34	PPFM34	24.97	18.17	4,054.37
35	PPFM35	23.37	19.13	4,250.00
36	PPFM36	24.33	19.60	4,037.78
37	PPFM37	23.20	18.97	3,877.02
38	PPFM38	22.35	19.70	3,912.22
39	PPFM39	22.02	19.60	3,221.33

40	PPFM40	21.55	17.57	3,817.84
41	PPFM41	23.65	15.47	3,545.07
42	PPFM42	24.34	21.33	4,385.67
43	PPFM43	19.17	22.00	3,537.80
44	PPFM44	20.95	21.17	3,786.82
45	PPFM45	23.67	21.97	3,867.93
46	PPFM46	22.08	15.87	3,986.13
47	PPFM47 (Reference strain)	23.03	18.90	3,943.45
48	Control	17.84	17.50	3,037.91
	CD (0.05)	2.908	1.338	365.251
	SEm (±)	1.03	0.48	129.90

*Mean of 3 replications

Table 8. Effect of PPFM isolates on shoot and root fresh weight of paddy seedlings

Sl. No.	Isolate code No.	Shoot fresh weight (g)/ seedling*	Root fresh weight (g)/ seedling*
1	PPFM1	0.51	0.25
2	PPFM2	0.45	0.21
3	PPFM3	0.54	0.30
4	PPFM4	0.54	0.28
5	PPFM5	0.53	0.26
6	PPFM6	0.65	0.33
7	PPFM7	0.57	0.28
8	PPFM8	0.67	0.30
9	PPFM9	0.63	0.29
10	PPFM10	0.56	0.25
11	PPFM11	0.54	0.25
12	PPFM12	0.52	0.26
13	PPFM13	0.54	0.23
14	PPFM14	0.62	0.27
15	PPFM15	0.60	0.27
16	PPFM16	0.60	0.35
17	PPFM17	0.57	0.22
18	PPFM18	0.55	0.25
19	PPFM19	0.58	0.30
20	PPFM20	0.60	0.29
21	PPFM21	0.61	0.30
22	PPFM22	0.58	0.29
23	PPFM23	0.55	0.24
24	PPFM24	0.66	0.30
25	PPFM25	0.57	0.31
26	PPFM26	0.57	0.33
27	PPFM27	0.56	0.27
28	PPFM28	0.52	0.25
29	PPFM29	0.55	0.29
30	PPFM30	0.60	0.31
31	PPFM31	0.50	0.24
32	PPFM32	0.57	0.28
33	PPFM33	0.65	0.30
34	PPFM34	0.54	0.27
35	PPFM35	0.58	0.34
36	PPFM36	0.54	0.24
37	PPFM37	0.57	0.30
38	PPFM38	0.46	0.23
39	PPFM39	0.61	0.29

40	PPFM40	0.59	0.25
41	PPFM41	0.61	0.27
42	PPFM42	0.67	0.32
43	PPFM43	0.49	0.23
44	PPFM44	0.42	0.26
45	PPFM45	0.65	0.34
46	PPFM46	0.56	0.26
47	PPFM47 (Reference strain)	0.54	0.28
48	Control	0.57	0.24
	CD (0.05)	0.096	0.050
	SEm (±)	0.03	0.02

*Mean of 3 replications

PPFM45, PPFM8, PPFM9, PPFM16, PPFM14, PPFM39, PPFM41 and PPFM30 which recorded shoot dry weight of 0.36, 0.35, 0.35, 0.34, 0.33, 0.33, 0.32, 0.31, 0.31 and 0.30 g respectively. All these treatments were statistically on par with PPFM42 and significantly superior to the control (0.27 g) (Table 9).

4.4.9 Dry Weight of Root (g)

The dry weight of root was significantly higher in seeds treated with PPFM (Table 9). Maximum dry weight of 0.20 g was recorded with the treatment PPFM42.This was followed by PPFM16 (0.19 g), PPFM35 (0.18 g), PPFM26 (0.17 g), PPFM 45 (0.17 g) and PPFM6 (0.17 g), which were statistically on par. These treatments were significantly superior to the control, which recorded a root dry weight of 0.09 g.

4.4.10 Root Shoot Ratio

The root shoot ratio of seedlings showed significant increase when seeds were treated with PPFM isolates (Table 9). Maximum root shoot ratio of 0.62 was observed when seeds were treated with PPFM26 and PPFM35. However, these treatments were statistically on par with the treatments PPFM3 (0.58) and PPFM16 (0.57). These treatments were significantly superior compared to control and reference strain which recorded root shoot ratio of 0.33 and 0.48 respectively.

4.5 EFFECT OF PPFM ISOLATES ON GROWTH AND YIELD OF PADDY.

4.5.1 Biometric Characters

The data on the effect of inoculation of PPFM isolates on growth characteristics of paddy are presented.

4.5.1.1 Plant Height (cm)

The data relating to the influence of PPFM isolates on plant height at various

Table 9. Effect of PPFM isolates on shoot dry weight, root dry weight and root shoot ratio of paddy seedlings

Sl. No.	Isolate code No.	Shoot dry weight (g)/ seedling*	Root dry weight (g)/ seedling*	RS Ratio
1	PPFM1	0.21	0.09	0.41
2	PPFM2	0.16	0.05	0.32
3	PPFM3	0.24	0.14	0.58
4	PPFM4	0.28	0.12	0.43
5	PPFM5	0.23	0.10	0.42
6	PPFM6	0.35	0.17	0.48
7	PPFM7	0.26	0.12	0.45
8	PPFM8	0.34	0.14	0.43
9	PPFM9	0.33	0.13	0.39
10	PPFM10	0.26	0.09	0.36
11	PPFM11	0.24	0.09	0.36
12	PPFM12	0.22	0.10	0.45
13	PPFM13	0.24	0.07	0.29
14	PPFM14	0.32	0.11	0.35
15	PPFM15	0.30	0.11	0.35
16	PPFM16	0.33	0.19	0.57
17	PPFM17	0.27	0.06	0.24
18	PPFM18	0.25	0.09	0.36
19	PPFM19	0.28	0.14	0.51
20	PPFM20	0.30	0.13	0.45
21	PPFM21	0.27	0.14	0.54
22	PPFM22	0.28	0.13	0.46
23	PPFM23	0.25	0.08	0.31
24	PPFM24	0.36	0.14	0.40
25	PPFM25	0.27	0.15	0.54
26	PPFM26	0.27	0.17	0.62
27	PPFM27	0.26	0.11	0.43
28	PPFM28	0.22	0.09	0.40
29	PPFM29	0.25	0.13	0.54
30	PPFM30	0.30	0.15	0.49
31	PPFM31	0.20	0.08	0.41
32	PPFM32	0.27	0.12	0.47
33	PPFM33	0.28	0.14	0.48
34	PPFM34	0.24	0.11	0.47
35	PPFM35	0.28	0.18	0.62
36	PPFM36	0.24	0.08	0.35
37	PPFM37	0.27	0.14	0.53
38	PPFM38	0.16	0.07	0.43
39	PPFM39	0.31	0.13	0.43

40	PPFM40	0.29	0.09	0.33
41	PPFM41	0.31	0.11	0.34
42	PPFM42	0.37	0.20	0.52
43	PPFM43	0.19	0.07	0.38
44	PPFM44	0.26	0.10	0.39
45	PPFM45	0.35	0.17	0.48
46	PPFM46	0.26	0.10	0.37
47	PPFM47 (Reference strain)	0.24	0.12	0.48
48	Control	0.27	0.09	0.33
	CD (0.05)	0.078	0.039	0.054
	SEm (±)	0.03	0.01	0.02

*Mean of 3 replications

stages of crop growth are presented in the Table 10.

At initial stage of transplanting *i.e.,* at 30 days after transplanting, the maximum plant height was recorded with inoculation of PPFM26 (51.85 cm) followed by PPFM 28 (50.65 cm) and PPFM21 (50.30 cm) which were statistically on par. The next best treatment in improving plant height was PPFM30 (49.25 cm) which was statistically on par with PPFM19 (48.35 cm). All these treatments were significantly superior to the control which recorded a plant height of 39.05 cm.

At 60 DAT, it was found that inoculation of PPFM26 recorded significantly higher plant height (72.80 cm) followed by PPFM28 (72.05 cm), PPFM 21 (71.40 cm), PPFM30 (71.20 cm), PPFM19 (71.15 cm), PPFM17 (70.75 cm), PPFM 18 (70.70 cm), and PPFM27 (70.50 cm). Here PPFM26 was statistically on par with others (Plate10). These treatments were found to be significantly superior to the control (60.10 cm).

At 90 DAT (at harvest), inoculation of PPFM26 (103.65 cm) was found to be the best among all treatments. The next best treatment in improving plant height were PPFM28 (102.35 cm), PPFM21 (101.70 cm), PPFM30 (101.05 cm), and PPFM19 (100.85 cm). PPFM26 was statistically on par with these treatments. However the control recorded a plant height of 84.55 cm (Plate 11).

4.5.1.2 Number of Tillers Hill^{-1}

The number of tillers hill^{-1} was significantly influenced by inoculation of PPFM. The crop has shown significant variation in tiller production at different growth stages.

At 30 DAT, plants inoculated with PPFM19 recorded significantly higher number of tillers hill^{-1} (25.00) followed by PPFM21 (24.67) and PPFM15 (23.00) which were statistically on par. All these treatments were found to be significantly

Table 10. Effect of PPFM isolates on height of paddy at various stages of crop growth

Sl. No.	Isolate code No.	Plant height (cm)		
		30 DAT	60 DAT	90 DAT
1	PPFM1	40.35	62.85	88.75
2	PPFM2	42.05	67.55	96.45
3	PPFM3	39.55	68.80	98.95
4	PPFM4	41.75	66.90	95.45
5	PPFM5	39.00	65.55	98.50
6	PPFM6	41.10	67.40	97.10
7	PPFM7	39.45	65.30	97.55
8	PPFM8	41.90	67.40	96.30
9	PPFM9	40.25	63.25	95.65
10	PPFM10	40.95	64.95	92.35
11	PPFM11	43.55	63.10	86.05
12	PPFM12	42.60	68.00	96.80
13	PPFM13	44.55	70.40	100.65
14	PPFM14	41.65	68.90	99.55
15	PPFM15	41.95	67.95	97.85
16	PPFM16	45.85	67.95	93.45
17	PPFM17	46.75	70.75	100.20
18	PPFM18	46.30	70.70	100.50
19	PPFM19	48.35	71.15	100.85
20	PPFM20	44.95	64.35	87.15
21	PPFM21	50.30	71.40	101.70
22	PPFM22	48.00	70.30	100.60
23	PPFM23	44.35	64.85	88.75
24	PPFM24	43.50	65.40	90.70
25	PPFM25	43.15	65.35	90.95
26	PPFM26	51.85	72.80	103.65
27	PPFM27	44.85	70.50	100.55
28	PPFM28	50.65	72.05	102.35
29	PPFM29	42.70	62.45	85.60
30	PPFM30	49.25	71.20	101.05
31	PPFM31	41.95	62.10	87.35
32	PPFM32	44.45	62.25	84.65
33	PPFM33	40.30	68.15	98.20

34	PPFM34	43.50	60.35	84.60
35	PPFM35	40.40	60.45	85.10
36	PPFM36	42.50	61.95	84.80
37	PPFM37	39.50	64.95	98.80
38	PPFM38	39.45	58.95	84.60
39	PPFM39	40.25	61.10	84.95
40	PPFM40	39.45	62.75	94.45
41	PPFM41	38.85	57.00	80.05
42	PPFM42	40.00	60.25	84.90
43	PPFM43	41.25	64.95	93.55
44	PPFM44	41.05	66.45	95.25
45	PPFM45	39.25	60.70	88.05
46	PPFM46	41.95	63.60	89.75
47	PPFM47 (Reference strain)	45.10	68.20	94.70
48	Control	39.05	60.10	84.55
	CD (0.05)	1.902	2.397	2.952
	SEm (±)	0.68	0.85	1.05

PFM26 Control PPFM28 Control PPFM21 Control

Plate 10. Effect of PPFM isolates on height of paddy at 60 DAT

PPFM26 Control PPFM26 Control PPFM26 Control

Plate 11. PPFM26 inoculated plants at 30, 60 and 90 DAT

superior to the control which recorded 14.33 tillers hill^{-1} (Plate 12).

At 60 DAT, plants inoculated with PPFM19 was found to be the best in tiller production (41.67) among all the treatments. This was statistically on par with other treatments PPFM21 (41.00), PPFM15 (40.67), PPFM4 (39.00) and PPFM7 (38.67). All these treatments were found to have significant effect on higher tiller production compared to control which recorded 24.00 tillers hill^{-1} (Plate 13).

At harvest stage (90 DAT), PPFM21 inoculated plants recorded the highest number of tillers hill^{-1} (32.67) followed by PPFM19 (31.67), PPFM15 (31.00) and PPFM4 (30.00) and these treatments were found to be statistically on par. These treatments recorded significantly superior tiller production as against control (14.67) (Plate 14) (Table 11).

4.5.1.3 Leaf Area Index

Significant increase in Leaf Area Index (LAI) was observed in plants inoculated with PPFMs (Table 12). The treatment PPFM19 recorded the maximum LAI of 4.81 and this was statistically on par with other treatments such as PPFM21 (4.78), PPFM28 (4.74), PPFM18 (4.68), PPFM6 (4.66), PPFM7 (4.64), PPFM1 (4.49), PPFM2 (4.48), PPFM4 (4.47) and the reference strain PPFM47 (4.38) as against the control which recorded a LAI of 3.80.

4.5.2 Yield and Yield Attributes

The result on the effect of PPFM isolates on yield and yield attributes are furnished here.

4.5.2.1 Number of Panicles Hill^{-1}

Table 11. Effect of PPFM isolates on tiller production of paddy at various stages of crop growth

Sl. No.	Isolate code No.	Number of tillers hill^{-1}		
		30 DAT	60 DAT	90 DAT
1	PPFM1	17.67	32.67	24.67
2	PPFM2	20.33	34.67	28.00
3	PPFM3	17.00	34.67	24.00
4	PPFM4	21.00	39.00	30.00
5	PPFM5	16.67	32.00	24.00
6	PPFM6	18.67	35.67	25.00
7	PPFM7	18.00	38.67	29.00
8	PPFM8	14.67	24.00	17.67
9	PPFM9	14.33	25.00	17.00
10	PPFM10	17.00	29.00	20.67
11	PPFM11	16.00	25.67	19.00
12	PPFM12	18.33	29.67	20.67
13	PPFM13	17.00	31.67	26.00
14	PPFM14	14.67	24.00	17.00
15	PPFM15	23.00	40.67	31.00
16	PPFM16	14.67	27.00	19.67
17	PPFM17	16.67	29.67	22.00
18	PPFM18	17.67	34.00	24.67
19	PPFM19	25.00	41.67	31.67
20	PPFM20	19.67	32.00	22.00
21	PPFM21	24.67	41.00	32.67
22	PPFM22	15.00	29.67	20.67
23	PPFM23	14.67	24.67	16.00
24	PPFM24	15.67	26.00	19.00
25	PPFM25	14.67	27.67	18.67
26	PPFM26	21.00	32.67	21.67
27	PPFM27	16.33	25.67	19.00
28	PPFM28	19.67	36.00	28.00
29	PPFM29	15.00	24.00	18.00
30	PPFM30	20.33	32.00	24.67
31	PPFM31	15.33	26.67	17.67
32	PPFM32	15.33	27.67	19.00
33	PPFM33	16.00	24.00	18.67
34	PPFM34	14.33	25.00	16.67
35	PPFM35	17.33	25.67	17.00
36	PPFM36	19.67	27.00	18.67

37	PPFM37	14.67	24.67	18.00
38	PPFM38	19.00	26.67	18.67
39	PPFM39	16.67	26.67	19.67
40	PPFM40	15.33	29.00	21.67
41	PPFM41	13.00	19.67	13.67
42	PPFM42	18.33	26.00	17.67
43	PPFM43	17.33	25.67	16.00
44	PPFM44	15.00	24.67	17.00
45	PPFM45	15.33	24.00	16.67
46	PPFM46	16.33	25.00	18.00
47	PPFM47 (Reference strain)	18.67	31.00	23.00
48	Control	14.33	24.00	14.67
	CD (0.05)	3.675	3.181	3.712
	SEm (±)	1.31	1.13	1.32

PPFM19 Control

PPFM21 Control

PPFM15 Control

Plate 12. Effect of PPFM isolates on tiller production of paddy at 30 days after transplanting

PPFM19 Control

PPFM21 Control

PPFM15 Control

Plate 13. Effect of PPFM isolates on tiller production of paddy at 60 days after transplanting

PPFM19 Control

PPFM21 Control

PPFM15 Control

Plate 14. Effect of PPFM isolates on tiller production of paddy at 90 days after transplanting

12. Effect of PPFM isolates on leaf area index of paddy

Sl. No.	Isolate code No.	LAI
1	PPFM1	4.49
2	PPFM2	4.48
3	PPFM3	4.36
4	PPFM4	4.47
5	PPFM5	4.38
6	PPFM6	4.66
7	PPFM7	4.64
8	PPFM8	3.81
9	PPFM9	3.95
10	PPFM10	3.89
11	PPFM11	3.90
12	PPFM12	4.49
13	PPFM13	4.52
14	PPFM14	3.81
15	PPFM15	4.56
16	PPFM16	4.28
17	PPFM17	4.20
18	PPFM18	4.68
19	PPFM19	4.81
20	PPFM20	4.57
21	PPFM21	4.78
22	PPFM22	4.45
23	PPFM23	3.88
24	PPFM24	4.12
25	PPFM25	4.37
26	PPFM26	4.63
27	PPFM27	4.04
28	PPFM28	4.74
29	PPFM29	3.81
30	PPFM30	4.59
31	PPFM31	4.20
32	PPFM32	4.36
33	PPFM33	4.09
34	PPFM34	3.96
35	PPFM35	4.04
36	PPFM36	4.28
37	PPFM37	3.91
38	PPFM38	4.20

39	PPFM39	4.20
40	PPFM40	4.45
41	PPFM41	3.89
42	PPFM42	3.88
43	PPFM43	3.85
44	PPFM44	3.81
45	PPFM45	3.83
46	PPFM46	3.90
47	PPFM47 (Reference strain)	4.38
48	Control	3.80
	CD (0.05)	0.527
	SEm (±)	0.19

The number of panicles hill^{-1} was significantly superior in plants inoculated with PPFM19 which recorded 33.00 panicles hill^{-1} compared to the other treatments and the control which recorded a panicle number of 14.67 hill^{-1}. The result on the effect of PPFM isolates on number of panicles hill^{-1} is presented in the Table 13.

4.5.2.2 Panicle Length (cm)

Maximum panicle length of 24.25 cm was recorded in plants inoculated with PPFM16 (Table 13). However, this was statistically on par with other treatments such as PPFM10, PPFM11, PPFM9, PPFM6, PPFM25, PPFM17, PPFM1, PPFM13, PPFM3 and PPFM29 which recorded panicle length of 24.20, 24.05, 23.85, 23.75, 23.70, 23.65, 23.50, 23.45, 23.15 and 23.10 cm respectively. These treatments were statistically superior to the control which recorded panicle length of 21.60 cm (Plate 15).

4.5.2.3 Number of Grains Panicle^{-1}

Number of grains panicle^{-1} was significantly influenced by PPFM treatment (Table 13). Inoculation of plants with PPFM10 recorded significantly higher number of grains panicle^{-1} (142.70) which was on par with treatments PPFM9 (136.00), PPFM11 (135.33), PPFM16 (134.00) and PPFM17 (130.80). All these treatments were found to be statistically superior to the control (99.33).

4.5.2.4 Number of Filled Grains Panicle^{-1}

The data indicated that filled grains panicle^{-1} was significantly influenced by PPFM inoculation (Table 13). Treatment with PPFM11 recorded significantly higher number of filled grains panicle^{-1}(131.00). This was found to be statistically on par with other treatments such as PPFM16, PPFM10, PPFM17, PPFM9, PPFM20, PPFM27, PPFM2 and PPFM18 which recorded 127.67, 127.33, 124.00, 122.67, 121.33, 120.33, 116.67 and 115.67 respectively. These treatments were found to be statistically superior compared to control (89.33).

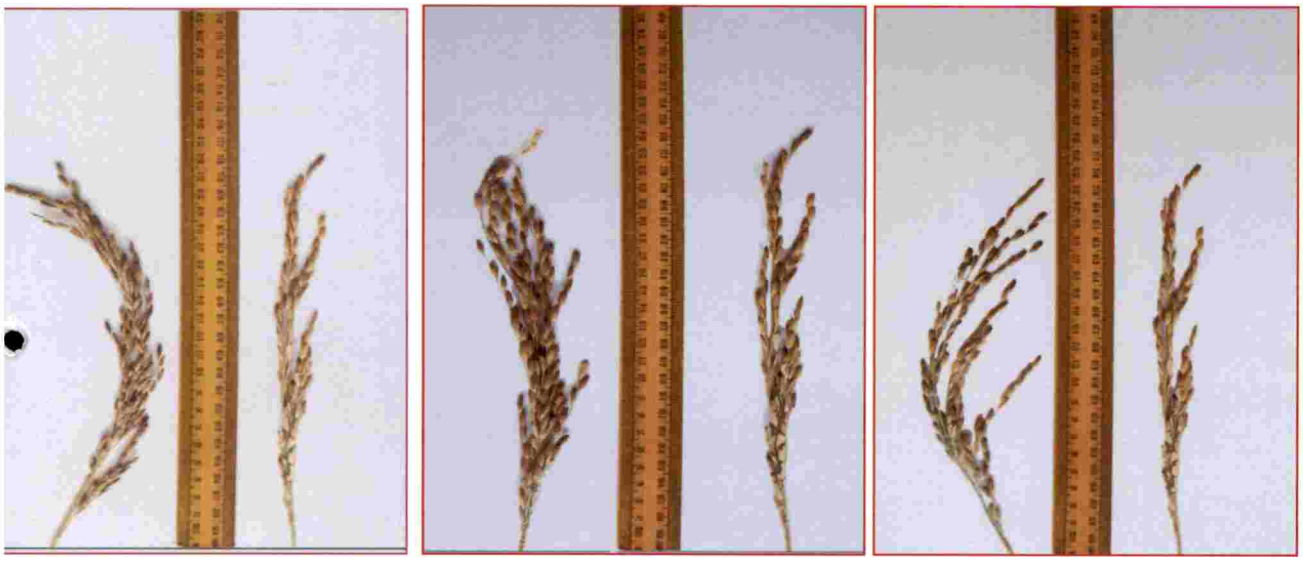
Table 13. Effect of PPFM isolates on yield and yield attributes of paddy at harvest

Sl. No.	Isolate code No.	Number of panicle hill^{-1}	Panicle length* (cm)	Number of grains panicle^{-1}*	Filled grains Panicle^{-1}*	Sterility percentage*
1	PPFM1	24.67	23.50	108.83	103.33	5.05
2	PPFM2	27.67	21.60	124.50	116.67	6.43
3	PPFM3	25.00	23.15	125.50	113.67	9.54
4	PPFM4	21.00	22.00	100.00	92.67	7.59
5	PPFM5	28.00	22.10	109.70	99.00	9.99
6	PPFM6	19.67	23.75	108.67	101.00	6.91
7	PPFM7	28.67	22.70	121.33	112.33	7.88
8	PPFM8	22.67	22.90	120.30	112.00	6.58
9	PPFM9	26.00	23.85	136.00	122.67	9.99
10	PPFM10	25.00	24.20	142.70	127.33	10.85
11	PPFM11	22.67	24.05	135.33	131.00	3.31
12	PPFM12	20.00	21.95	115.00	108.67	5.73
13	PPFM13	21.00	23.45	110.17	103.00	6.27
14	PPFM14	19.67	21.85	101.83	93.33	8.75
15	PPFM15	27.67	22.95	104.20	98.33	5.74
16	PPFM16	24.00	24.25	134.00	127.67	4.87
17	PPFM17	22.67	23.65	130.80	124.00	4.98
18	PPFM18	25.00	22.90	124.00	115.67	6.85
19	PPFM19	33.00	22.50	111.80	102.67	4.08
20	PPFM20	23.67	22.95	126.20	121.33	3.95
21	PPFM21	24.00	21.90	108.00	98.00	9.24
22	PPFM22	23.67	21.95	118.70	111.33	6.27
23	PPFM23	20.67	21.80	100.37	88.67	11.43
24	PPFM24	24.00	22.20	83.00	68.67	17.48
25	PPFM25	19.67	23.70	87.50	83.67	4.41
26	PPFM26	23.67	22.35	104.20	97.00	6.97
27	PPFM27	26.00	22.05	124.33	120.33	3.12
28	PPFM28	30.00	22.25	99.83	88.33	11.37
29	PPFM29	21.67	23.10	115.17	108.00	6.08
30	PPFM30	16.67	22.80	106.50	102.00	4.25
31	PPFM31	21.67	22.30	117.17	110.67	5.55
32	PPFM32	19.00	22.30	101.30	75.67	8.15
33	PPFM33	18.67	22.10	108.70	106.33	2.31
34	PPFM34	28.67	21.60	107.67	101.00	5.99
35	PPFM35	24.67	21.25	110.50	103.67	6.33
36	PPFM36	16.00	22.05	110.00	99.67	9.56
37	PPFM37	23.67	23.15	112.00	106.00	5.29
38	PPFM38	19.67	21.90	117.67	111.00	5.50

39	PPFM39	15.00	21.60	117.30	113.00	3.41
40	PPFM40	20.67	18.90	102.83	72.33	13.49
41	PPFM41	15.00	22.40	99.60	96.67	10.32
42	PPFM42	18.00	22.70	106.33	98.00	7.99
43	PPFM43	22.00	21.80	107.50	102.67	4.78
44	PPFM44	22.00	21.80	114.50	109.00	4.76
45	PPFM45	23.67	22.50	90.70	83.33	8.22
46	PPFM46	26.67	22.25	115.70	102.33	11.71
47	PPFM47 (Reference strain)	23.67	21.85	100.20	89.33	11.00
48	Control	14.67	21.60	99.33	89.33	10.12
	CD (0.05)	2.524	1.175	15.701	16.781	4.192
	SEm (±)	0.90	0.42	5.58	5.97	1.49

*Mean of 5 replications

PPFM16 Control PPFM10 Control PPFM11 Control



PPFM9 Control PPFM6 Control PPFM25 Control

Plate 15. Effect of PPFM isolates on panicle length of paddy at harvest

4.5.2.5 Sterility Percentage

Results revealed that sterility percentage was significantly influenced by PPFM treatments (Table 13). PPFM33 recorded the least sterility percentage of 2.31. This was on par with other treatments PPFM27 (3.12 %), PPFM11 (3.31 %), PPFM39 (3.41 %) and PPFM30 (4.25 %). These treatments were statistically superior to the control which recorded sterility percentage of 10.12 per cent.

4.5.2.6 Thousand Grain Weight (g)

The results indicated that thousand grain weight was significantly influenced by the PPFM treatments. Among all the treatments, PPFM16 recorded significantly higher thousand grain weight (30.66 g) which was on par with PPFM25 (28.97 g). These treatments were statistically superior to the control which recorded a thousand grain weight of 24.28 g. The thousand grain weight of the reference strain was 26.13 g (Table 14).

4.5.2.7 Grain Yield Hill^{-1}

Inoculation of PPFM significantly increased the yield of paddy. PPFM11 recorded the highest yield of 46.30 g hill^{-1} and this was statistically on par with PPFM16 (45.05 g hill^{-1}), PPFM6 (44.75 g hill^{-1}), PPFM18 (43.95 g hill^{-1}), PPFM15 (43.25 g hill^{-1}), PPFM19 (42.95 g hill^{-1}), and PPFM9 (42.05 g hill^{-1}) whereas the control recorded a grain yield of 33.65 g hill^{-1}. Only 38.40 g hill^{-1} of grain yield was obtained in plants treated with reference strain (Table 14).

4.5.2.8 Straw Yield Hill^{-1}

The straw yield was significantly higher in plants treated with PPFM isolates. The isolate PPFM29 recorded the highest straw yield of 75.10 g hill^{-1} followed by PPFM20 (73.50 g hill^{-1}), PPFM30 (71.60 g hill^{-1}), PPFM2 (71.50 g hill^{-1}) and PPFM21 (70.40 g hill^{-1}). The control plants recorded a straw yield of 51.40 g hill^{-1} and the reference strain 65.00 g hill^{-1} (Table 14).

4.5.2.9 Dry Matter Production Hill^{-1} (at Harvest)

Analysis of the data on table 14 indicated that there was significant effect on dry matter production of paddy treated with PPFMs. Maximum dry matter production (136.70 g hill^{-1}) was recorded in plants inoculated with PPFM35, which were followed by PPFM37 (135.20 g hill^{-1}) and PPFM36 (133.80 g hill^{-1}) which were statistically on par, whereas, control recorded a dry matter production of 73.93 g hill^{-1}.

4.5.3 Disease Incidence

No incidence of disease was noticed on plants during the period of pot culture studies.

4.5.4 Biochemical Parameters

The results on the effect of PPFM isolates on biochemical parameters of plant are presented.

4.5.4.1 Chlorophyll Content

The chlorophyll content of the plants significantly differed due to various inoculation treatments.

At 60 DAT, the maximum chlorophyll content of 1.97 µg g sample tissue^{-1} was recorded in PPFM46 and PPFM18 and these were statistically on par with treatments such as PPFM12 (1.90 µg g^{-1}), PPFM11 (1.85 µg g^{-1}), PPFM16 (1.85 µg g^{-1}), PPFM42 (1.84 µg g^{-1}), PPFM2 (1.82 µg g^{-1}), the reference strain PPFM47 (1.80 µg g^{-1}), PPFM26 (1.79 µg g^{-1}), PPFM35 (1.77 µg g^{-1}), PPFM45 (1.77 µg g^{-1}), PPFM33 (1.75 µg g^{-1}), PPFM1 (1.73 µg g^{-1}), PPFM41 (1.73 µg g^{-1}) and PPFM8 (1.72 µg g^{-1}). All these treatments were found to be significantly superior

Table 14. Effect of PPFM isolates on yield and yield attributes of paddy at harvest

Sl. No.	Isolate code No.	1000 grain weight (g)	Grain yield hill^{-1} (g)	Straw yield hill^{-1} (g)	Dry matter production (g/hill)
1	PPFM1	24.37	35.10	61.20	86.60
2	PPFM2	27.33	38.05	71.50	97.00
3	PPFM3	26.06	39.95	65.50	91.80
4	PPFM4	26.83	38.10	52.90	89.30
5	PPFM5	26.41	34.55	57.00	99.40
6	PPFM6	27.15	44.75	61.70	91.80
7	PPFM7	26.58	41.85	52.10	93.80
8	PPFM8	23.85	35.65	54.40	74.40
9	PPFM9	26.10	42.05	54.50	74.90
10	PPFM10	26.00	40.15	56.90	87.70
11	PPFM11	26.28	46.30	58.50	89.90
12	PPFM12	25.05	40.40	64.50	99.70
13	PPFM13	24.31	35.05	53.50	85.20
14	PPFM14	27.74	40.85	53.50	94.40
15	PPFM15	25.13	43.25	66.80	74.70
16	PPFM16	30.66	45.05	58.50	88.10
17	PPFM17	27.92	41.05	53.90	74.40
18	PPFM18	27.52	43.95	70.10	128.00
19	PPFM19	28.44	42.95	64.00	101.20
20	PPFM20	25.82	38.30	73.50	133.00
21	PPFM21	26.34	36.25	70.40	111.10
22	PPFM22	25.86	38.40	68.80	126.50
23	PPFM23	27.85	35.65	52.30	89.60
24	PPFM24	28.33	38.20	57.80	108.00
25	PPFM25	28.97	33.85	54.40	83.50
26	PPFM26	26.48	38.20	63.00	106.30
27	PPFM27	28.69	40.60	60.70	99.30
28	PPFM28	24.26	33.85	53.90	80.50
29	PPFM29	26.51	33.60	75.10	84.80
30	PPFM30	26.97	34.60	71.60	129.10
31	PPFM31	27.82	30.30	52.60	78.30
32	PPFM32	26.59	39.45	59.30	111.20
33	PPFM33	24.97	35.65	54.70	88.80
34	PPFM34	27.28	36.05	45.20	85.70
35	PPFM35	26.89	33.70	67.00	136.70
36	PPFM36	28.71	34.55	66.20	133.80
37	PPFM37	25.95	37.85	62.70	135.20
38	PPFM38	24.22	35.80	53.50	131.30

39	PPFM39	26.44	33.60	51.60	81.50
40	PPFM40	25.27	35.15	60.20	81.93
41	PPFM41	24.77	29.55	44.20	78.70
42	PPFM42	24.86	33.80	54.70	101.60
43	PPFM43	24.48	34.15	66.20	131.90
44	PPFM44	25.08	35.10	51.50	85.00
45	PPFM45	26.33	33.85	60.50	82.30
46	PPFM46	24.35	31.15	55.70	87.90
47	PPFM47 (Reference strain)	26.13	38.40	65.00	119.30
48	Control	24.28	33.65	51.40	73.93
	CD (0.05)	1.749	4.245	4.754	3.645
	SEm (±)	0.62	1.51	1.69	1.30

to the control which recorded a total chlorophyll content of 1.40 µg g^{-1} of sample tissue (Table 15).

4.5.4.2 Proline Content

Inoculation of PPFM isolates showed significant increase in proline content over the control. The details are presented in Table 16.

Proline content of the plants inoculated with PPFM ranged between 5.09 to 285.51 µ moles g $tissue^{-1}$. Plants inoculated with PPFM10 recorded the maximum value of 285.51 µmoles g $tissue^{-1}$, which was on par with proline content (285.04 µmoles g $tissue^{-1}$) of plants inoculated with PPFM19. These treatments were significantly superior to control which recorded a proline content of 36.79 µmoles g $tissue^{-1}$.

4.5.5.3 Cell Membrane Stability Index

The data on the effect of inoculation of PPFM isolates on plant cell membrane stability index are presented in Table 16.

Data showed that PPFM treatments had significant influence on plant cell membrane stability. A significantly higher cell membrane stability index of 80.83% was recorded with PPFM28, which was statistically on par with PPFM32, PPFM43, PPFM36, PPFM29, PPFM31, PPFM41, PPFM37, PPFM33, PPFM46, PPFM39, PPFM45, PPFM47 (reference strain), PPFM44, PPFM14, PPFM30, PPFM40, PPFM38, PPFM1 and PPFM42 which recorded cell membrane stability index of 80.70, 80.67, 80.10, 79.70, 79.70, 79.53, 79.43, 79.40, 79.36, 79.33, 79.10, 79.10, 79.06, 78.97, 78.87, 78.43, 78.13, 77.90 and 77.80 per cent respectively.

Calculation of Weighted Average Ranks

As none of the treatments showed superior performance for all the parameters evaluated, the five treatments having top weighted average ranks were

Table 15. Effect of PPFM isolates on chlorophyll content of paddy content at 60 DAT

Sl. No.	Isolate code No.	Chlorophyll a (mg g tissue^{-1})	Chlorophyll b (mg g tissue^{-1})	Total Chlorophyll (mg g tissue^{-1})
1	PPFM1	0.93	0.79	1.73
2	PPFM2	0.96	0.86	1.82
3	PPFM3	0.94	0.39	1.43
4	PPFM4	0.99	0.72	1.71
5	PPFM5	0.90	0.75	1.65
6	PPFM6	0.90	0.72	1.62
7	PPFM7	0.87	0.59	1.46
8	PPFM8	0.96	0.76	1.72
9	PPFM9	0.73	0.52	1.45
10	PPFM10	0.98	0.70	1.68
11	PPFM11	1.00	0.86	1.85
12	PPFM12	1.01	0.90	1.90
13	PPFM13	0.91	0.68	1.59
14	PPFM14	0.91	0.71	1.62
15	PPFM15	0.95	0.68	1.63
16	PPFM16	1.08	0.77	1.85
17	PPFM17	0.91	0.68	1.59
18	PPFM18	1.05	0.92	1.97
19	PPFM19	0.95	0.77	1.72
20	PPFM20	0.86	0.64	1.49
21	PPFM21	0.67	0.48	1.45
22	PPFM22	0.81	0.56	1.46
23	PPFM23	0.85	0.57	1.42
24	PPFM24	0.79	0.52	1.41
25	PPFM25	0.74	0.48	1.42
26	PPFM26	0.99	0.80	1.79
27	PPFM27	0.98	0.71	1.69
28	PPFM28	0.85	0.59	1.44
29	PPFM29	0.91	0.68	1.58
30	PPFM30	0.77	0.54	1.41
31	PPFM31	0.93	0.73	1.66
32	PPFM32	0.93	0.77	1.70
33	PPFM33	0.96	0.80	1.75

34	PPFM34	0.86	0.68	1.54
35	PPFM35	1.02	0.75	1.77
36	PPFM36	0.87	0.62	1.49
37	PPFM37	0.92	0.67	1.59
38	PPFM38	0.95	0.76	1.71
39	PPFM39	0.81	0.68	1.49
40	PPFM40	0.90	0.67	1.58
41	PPFM41	0.95	0.77	1.73
42	PPFM42	0.98	0.87	1.84
43	PPFM43	0.89	0.74	1.63
44	PPFM44	0.96	0.72	1.68
45	PPFM45	1.03	0.74	1.77
46	PPFM46	1.04	0.93	1.97
47	PPFM47 (Reference strain)	1.02	0.78	1.80
48	Control	0.88	0.52	1.40
	CD (0.05)	0.113	0.171	0.258
	SEm (±)	0.04	0.06	0.09

Table 16. Effect of PPFM isolates on proline content and cell membrane stability index of paddy at 60 DAT

Sl. No.	Isolate code No.	Proline content (μ moles g tissue^{-1})	Cell membrane stability index (%)
1	PPFM1	30.61	77.90
2	PPFM2	149.84	72.30
3	PPFM3	11.20	64.10
4	PPFM4	30.24	72.23
5	PPFM5	59.86	75.97
6	PPFM6	82.30	74.77
7	PPFM7	21.86	66.83
8	PPFM8	78.93	74.23
9	PPFM9	164.81	77.30
10	PPFM10	285.51	71.50
11	PPFM11	248.39	66.80
12	PPFM12	40.16	73.13
13	PPFM13	118.43	64.80
14	PPFM14	224.89	78.97
15	PPFM15	261.39	68.07
16	PPFM16	270.76	71.37
17	PPFM17	8.09	68.23
18	PPFM18	29.98	66.20
19	PPFM19	285.04	63.17
20	PPFM20	5.09	64.17
21	PPFM21	11.24	67.90
22	PPFM22	175.32	73.53
23	PPFM23	24.78	72.40
24	PPFM24	167.60	69.83
25	PPFM25	23.98	72.70
26	PPFM26	77.50	64.80
27	PPFM27	187.84	76.97
28	PPFM28	263.51	80.83
29	PPFM29	200.54	79.70
30	PPFM30	58.21	78.87
31	PPFM31	33.42	79.70
32	PPFM32	99.58	80.70
33	PPFM33	51.51	79.40
34	PPFM34	123.89	69.16 7
35	PPFM35	29.14	75.10

36	PPFM36	38.44	80.10
37	PPFM37	104.19	79.43
38	PPFM38	68.09	78.13
39	PPFM39	39.52	79.33
40	PPFM40	55.04	78.43
41	PPFM41	55.52	79.53
42	PPFM42	36.87	77.80
43	PPFM43	62.86	80.67
44	PPFM44	56.95	79.06
45	PPFM45	13.01	79.10
46	PPFM46	30.55	79.36
47	PPFM47 (Reference strain)	139.10	79.10
	Control	36.79	
	C.D (0.05)	12.832	3.286
	SEm (±)	4.56	1.17

selected for further characterization. Growth promotion efficacy of the isolates was also considered before finalizing the five efficient isolates. The isolates having top weighted average ranks were PPFM11, PPFM16, PPFM19, PPFM22 and PPFM35.

4.5.5 Influence of Selected PPFM Isolates on Growth and Yield of Paddy

The selected isolates significantly improved the seed germination and seedling growth of paddy compared to uninoculated control and reference strain. Also, these selected isolates significantly enhanced the growth, yield and yield attributes of paddy (Table 17).

The per cent increase in seedling root shoot ratio due to inoculation with selected PPFM strains ranged from 9.09 to 87.88 per cent against uninoculated control (Table 18).

Significant increase in growth parameters was observed in plants treated with selected PPFM strains. They improved plant height and tiller production compared to control plants (Table 18).

Seeds treated with these selected PPFM isolates recorded 9.30 to16.28 per cent increase in seed germination over control and 1.06 to 6.38 per cent increase over reference strain (Table 19).

The per cent increase in seedling vigour index due to the application of selected PPFM isolates ranged from 37.66 to 56.57 over control. These isolates also had significant effect on seedling vigour index (SVI) compared to reference strain, which recorded 6.05 to 20.61 per cent increase in SVI over reference strain (Table 19).

Selected PPFM isolates recorded 0.15 to 37.59 per cent yield increase over control and these isolates were superior to reference strain with 11.85 to 20.57 per cent increase of grain yield over reference strain (Table 19).

The effect of selected PPFM isolates growth and yield of paddy are represented in Plates 16, 17 and 18.

Table 17. Effect of selected PPFM isolates growth and yield of paddy

Sl. No.	Isolate	Seed germination (%)	Seedling Vigour Index	RS Ratio	Plant height (cm) (60DAT)	Number of tillers $hill^{-1}$ (60 DAT)	1000 grain weight (g)	Grain yield $hill^{-1}$ (g)
1	PPFM11	98	4646.83	0.36	63.10	25.67	26.28	46.30
2	PPFM16	94	4465.37	0.57	67.95	27.00	30.66	45.05
3	PPFM19	95	4181.97	0.51	71.15	41.67	28.44	42.95
4	PPFM22	96	4756.35	0.46	70.30	29.67	25.86	38.40
5	PPFM35	100	4250.00	0.62	60.45	25.67	26.89	33.70
6	PPFM47 (Reference strain)	94	3943.45	0.48	68.20	31.00	26.13	38.40
7	Control	86	3037.91	0.33	60.10	24.00	24.28	33.65

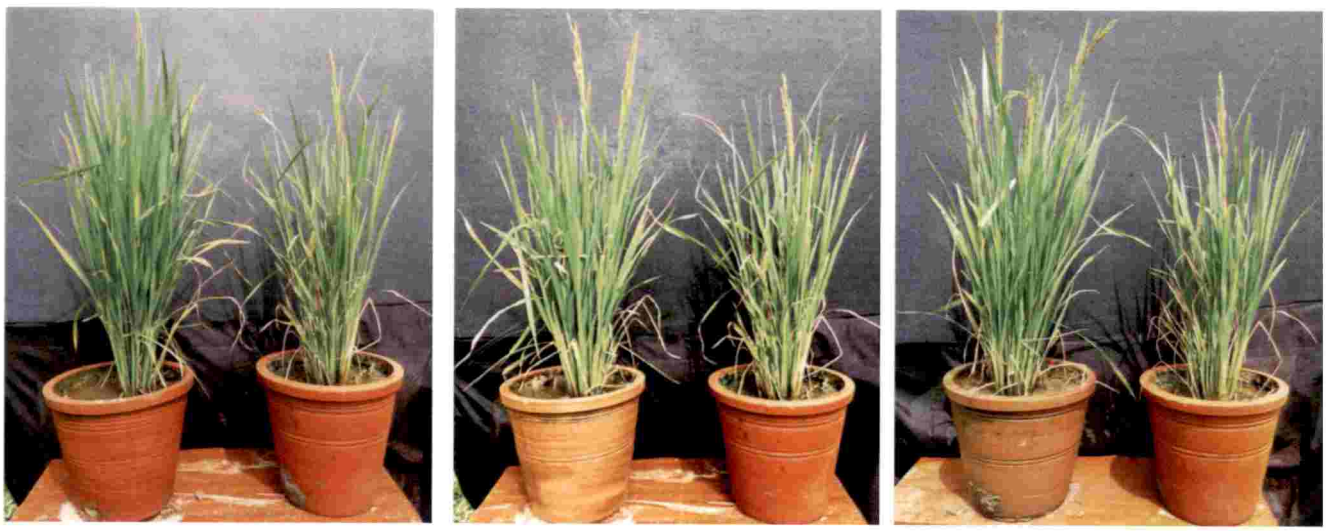
Table 18. Per cent increase in growth parameters of paddy over the control by the selected PPFM isolates

Sl. No.	Isolate	Per cent increase in root shoot ratio	Per cent increase in plant height (60DAT)	Per cent increase in number of tillers hill^{-1} (60 DAT)
1	PPFM11	9.09	4.99	6.96
2	PPFM16	72.73	13.06	12.50
3	PPFM19	54.55	18.39	73.63
4	PPFM22	39.39	16.97	23.63
5	PPFM35	87.88	0.58	6.96

Table 19. Per cent increase in seed germination, seedling vigour index and grain yield of paddy over the control and reference strain by the selected PPFM isolates

Sl. No.	Isolate	Per cent increase in seed germination		Per cent increase in seedling vigour index		Per cent increase in grain yield hill^{-1}	
		Over control	Over reference strain	Over control	Over reference strain	Over control	Over reference strain
1	PPFM11	13.95	4.26	52.96	17.84	37.59	20.57
2	PPFM16	9.30	0	46.99	13.24	33.88	17.32
3	PPFM19	10.47	1.06	37.66	6.05	27.64	11.85
4	PPFM22	11.63	2.13	56.57	20.61	14.12	0
5	PPFM35	16.28	6.38	39.90	7.77	0.15	0

PPFM11 Control PPFM16 Control PPFM19 Control



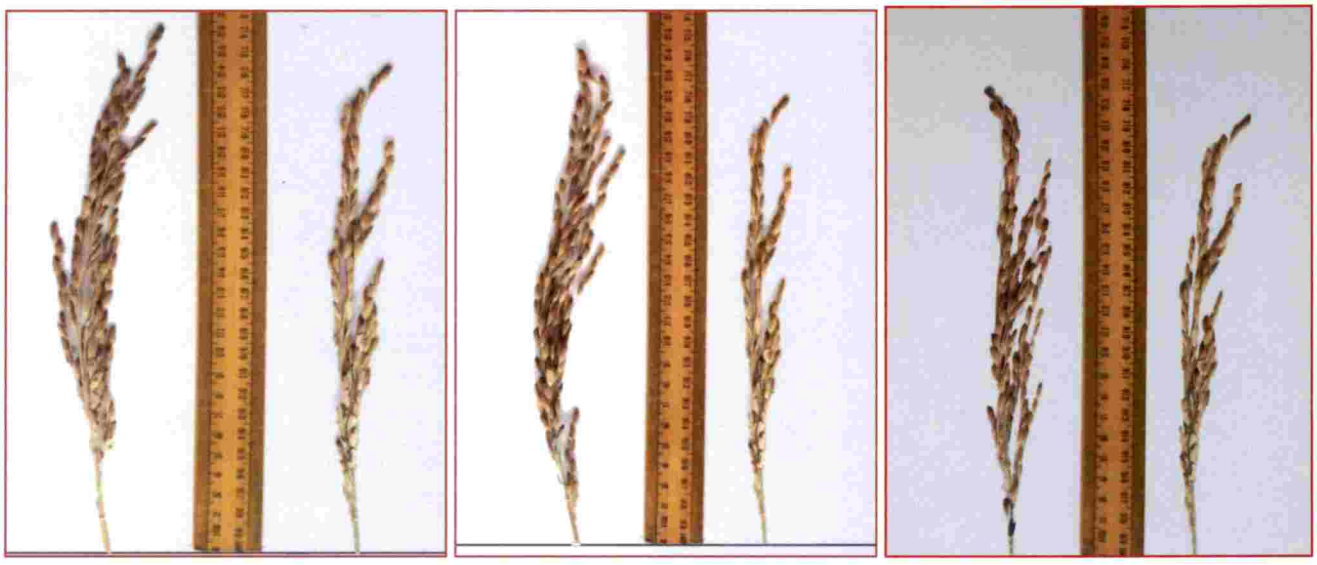
PPFM22 Control PPFM35 Control PPFM47(R. strain) Control

Plate 16. Plants treated with selected PPFM isolates at 60 days after transplanting

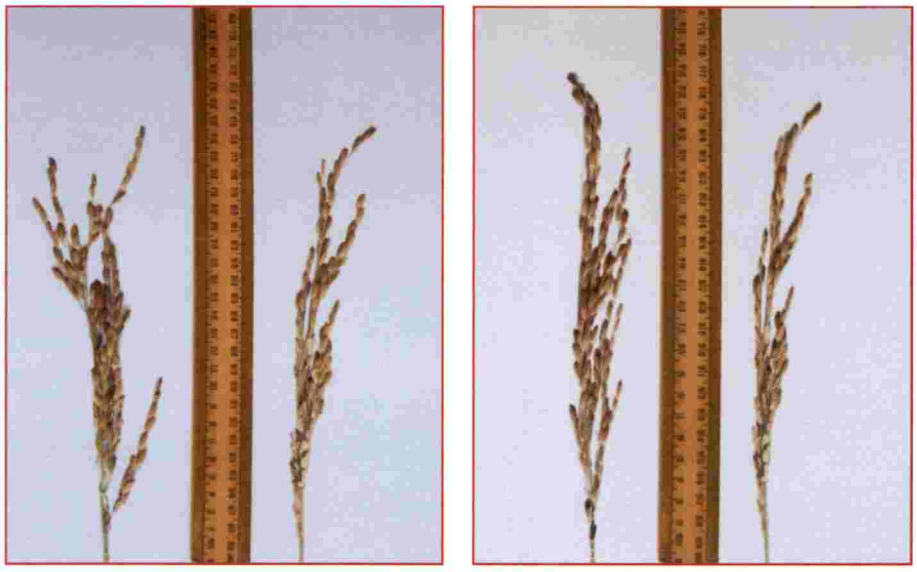
PPFM11 Control PPFM16 Control PPFM19 Control

PPFM22 Control PPFM35 Control

Plate 17. Effect of selected PPFM isolates on shoot and root growth of paddy seedlings



PPFM11 Control PPFM16 Control PPFM19 Control



PPFM22 Control PPFM35 Control

Plate 18. Effect of selected PPFM isolates on panicle length of paddy at harvest

4.6 CHARACTERIZATION OF ISOLATES OF PPFM

The superior isolates PPFM11, PPFM16, PPFM19, PPFM22 and PPFM35 were characterized based on morphological, biochemical and molecular characters.

4.6.1 Morphological Characterization of Selected PPFM isolates

The superior isolates selected were subjected to morphological characterization and the results are presented in Table 20. The results revealed that all the isolates were rod shaped, stained gram negative and exhibited motility.

Further one week after incubation, pink colonies with different color intensities were observed on AMS medium. Out of five efficient isolates, two isolates exhibited pale pink colored colonies, two isolates exhibited medium pink and remaining one isolate was found to be of dark pink color. The colony morphology of these isolates is represented in Plate 19.

4.6.2 Biochemical Characterization of Selected PPFM isolates

For further characterization, these five selected isolates were subjected to a series of biochemical tests. Using the results of various biochemical tests, a tentative genus level identification was done. All the 5 isolates were identified to be belonging to genus *Methylobacterium.* The results are presented in Table 21. All the isolates confirmed to be negative for Methyl red, Voges-Proskauer test, Phenylalanine deamination, H_2S production, Arginine lyase utilization and positive for Oxidase, Urease, Catalase activity, Indole production, Citrate, Lysine and Malonate utilization. None of the isolates could reduce nitrate to nitrite (Plate 20).

4.6.2.1 Utilization of Different Carbon Substrates by Selected PPFM Isolates

The utilization pattern of different carbon substrates by PPFM isolates are presented in Table 22 (Plate 21).

The methylotrophic bacteria having capability to grow on different C1 compounds as sole source of carbon and energy, can also grow on wide range of

Table 20. Morphological characterization of selected PPFM isolates

Sl. No.	Isolate code No.	Cell shape	Motility	Gram reaction	Pigmentation
1	PPFM11	Rod	Positive	Negative	Dark Pink
2	PPFM16	Rod	Positive	Negative	Pale pink
3	PPFM19	Rod	Positive	Negative	Pale pink
4	PPFM22	Rod	Positive	Negative	Medium pink
5	PPFM35	Rod	Positive	Negative	Medium pink

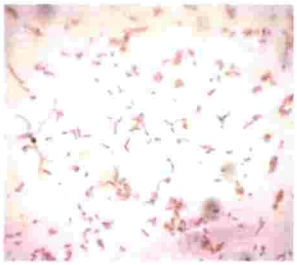
Table 21. Biochemical characterization of selected PPFM isolates

Sl. No	Biochemical Tests	PPFM11	PPFM16	PPFM19	PPFM22	PPFM35
1	Citrate utilization	+	+	+	+	+
2	Lysine utilization	+	+	+	+	+
3	Ornithine utilization	+	-	+	+	+
4	Urease	+	+	+	+	+
5	Phenylalanine deamination	-	-	-	-	-
6	H_2S production	-	-	-	-	-
7	Nitrate reduction	-	-	-	-	-
8	Catalase	+	+	+	+	+
9	Arginine lyase	-	-	-	-	-
10	Malonate utilization	+	+	+	+	+
11	VogesProskauer	-	-	-	-	-
12	Indole	+	+	+	+	+
13	Oxidase	+	+	+	+	+
14	Methyl red	-	-	-	-	-

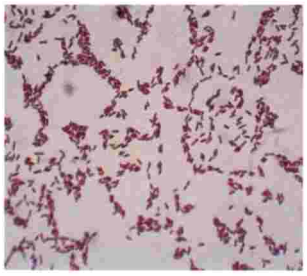
Table 22. Utilization of different carbon substrates by selected PPFM isolates

Sl. No	Carbon Substrate	PPFM11	PPFM16	PPFM19	PPFM22	PPFM35
1	D- Glucose	+	+	+	+	+
2	D- Fucose	-	+	-	-	-
3	D- Xylose	-	+	-	-	-
4	L- Arabinose	+	+	-	-	-
5	D- Fructose	+	-	+	+	+
6	L- Aspartate/ L- Glutamate	-	+	-	+	-
7	Sebacate	-	-	-	-	-
8	Acetate	+	+	+	+	+
9	Betaine	+	-	+	+	+
10	Tartarate	-	-	+	+	+
11	Ethanol	+	+	+	+	+
12	Methylamine	-	-	+	+	-
13	Dimethylamine	+	-	+	+	+
14	Formaldehyde	+	+	+	+	+
15	Glycerol	+	+	+	+	+
16	Methanol	+	+	+	+	+
17	Formate	-	+	+	+	+
18	Succinate	-	+	+	-	-
19	Lactate	+	+	+	+	+
20	Pyruvate	+	+	+	+	+
21	Salicylate	-	-	-	+	+
22	Nutrient agar	+	+	+	+	+
23	Fumarate	+	+	+	+	+
24	Rhamnose	-	-	-	-	-
25	Raffinose	-	-	-	+	-
26	Esculine	-	-	-	-	-
27	Cellobiose	-	-	-	-	-
28	Melibiose	-	-	-	+	-
29	Saccharose	-	-	-	+	-

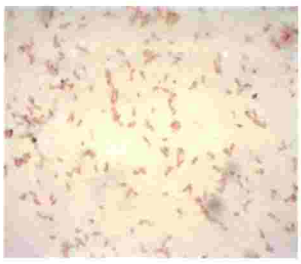
PPFM11



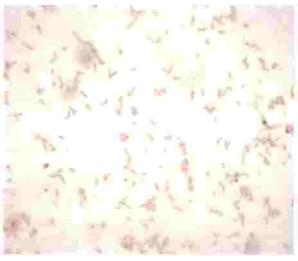
PPFM16



PPFM19



PPFM22



PPFM35

Plate 19. Colony morphology of the selected PPFM isolates and Gram reaction

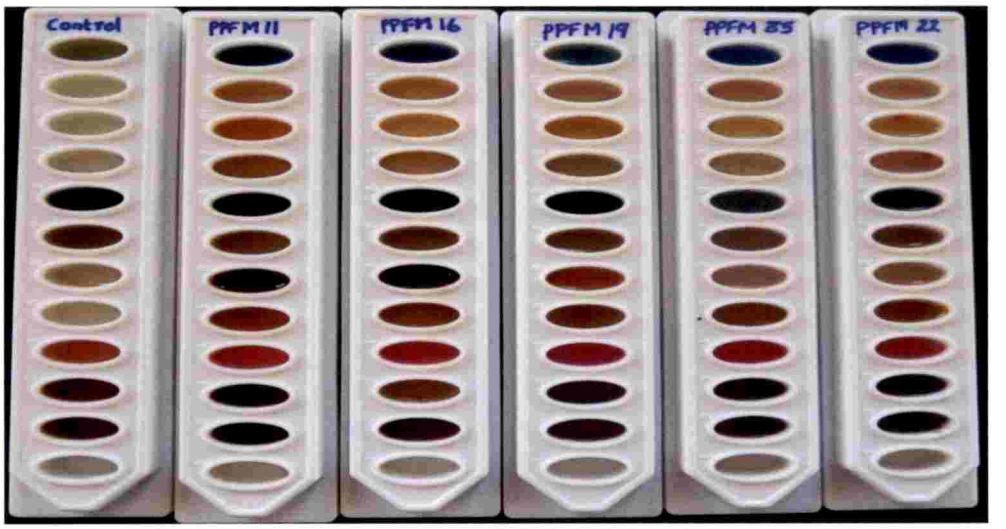


Plate 20. Biochemical characterization of selected PPFM isolates

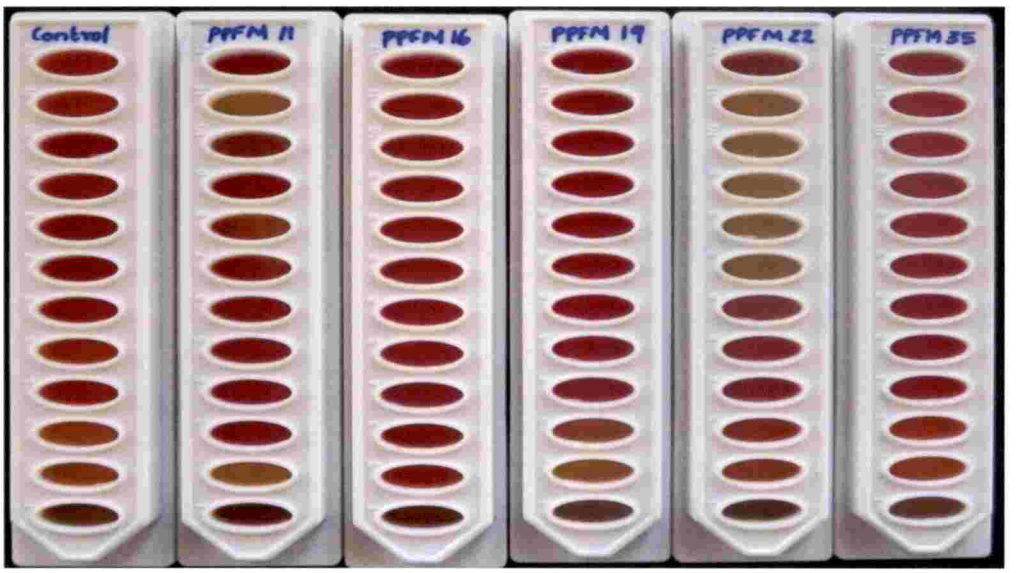


Plate 21. Carbohydrate utilization tests for selected PPFM isolates

multi carbon growth substrates making them facultatively methylotrophic. All the selected isolates were tested for the utilization of the 29 different carbon compounds and they showed wide variability in carbon utilization pattern.

4.6.3 Molecular Characterization of Selected Isolates PPFM isolates

The results of BLAST search details of the sequences producing most significant alignment of the bacterial isolates are presented in Table 23. The isolates PPFM11 and PPFM16 were found to be very close to *M. aquaticum* and *M. radiotolerans* respectively whereas PPFM19, PPFM22 and PPFM35 were found to be very close to *M. populi.* 16S rRNA sequence of bacterial isolates obtained with universal primer is presented in Table 24.

Table 23. BLAST search details of the sequences producing most significant alignment of the bacterial isolates

Isolate	Description	Max score	Total score	Query cover (%)	E value	Identity (%)	Accession no.
PPFM 11	*Methylobacterium aquaticum* DNA, complete genome, strain: MA-22A	2145	21221	100	0.0	99	AP 014704.1
PPFM 16	*Methylobacterium radiotolerans strain* VRI6-3 16S ribosomal RNA gene, partial sequence	1951	1951	100	0.0	97	KY 882067.1
PPFM 19	*Methylobacterium populi* strain BJ001 16S ribosomal RNA, partial sequence	2207	2207	100	0.0	99	NR 074257.1
PPFM 22	*Methylobacterium populi* BJ001, complete genome	2159	10771	100	0.0	100	NC 010725.1
PPFM 35	*Methylobacterium populi* strain MSSRF_1B39 16S ribosomal RNA gene, partial sequence	2226	2226	100	0.0	100	MG 597196.1

Table 24. 16S rRNA sequence of bacterial isolates obtained with universal primer

Isolate	SEQUENCE
PPFM11	ACGCGTGGGAACGTGCCCTTCGGTTCGGAATAACTCAG GGAAACTTGAGCTAATACCGGATACGTGCGAGAGCAG AAAGGTTTACTGCCGAAGGATCGGCCCGCGTCTGATTA GCTAGTTGGTGAGGTTACGGCTCACCAAGGCGACGATC AGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGA CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT GGGGAATATTGGACAATGGGGGCAACCCTGATCCAGC CATGCCGCGTGAGTGATGACGGCCTTAGGGTTGTAAAG CTCTTTTTCTCCGGGACGATAATGACGGTACCGGAGGA ATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAA TACGAAGGGGGCTAGCGTTGCTCGGAATCACTGGGCGT AAAGGGCGCGTAGGCGGCTGATTTAGTCGAGGGTGAA AGCCCGTGGCTCAACCACGGAATGGCCTTCGATACTGG TTGGCTTGAGACCGGAAGAGGACAGCGGAACTGCGAG TGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACCA GTGGCGAAGGCGGCTGTCTGGTCCGGTTCTGACGCTGA GGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTGGTAGTCCACGCTGTAAACGATGAATGCTAGCCGTT GGGGTGCATGCACCTCAGTGGCGCCGCTAACGCATTAA GCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT CAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC ATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCA TCCCTTGACATGGCATGCGAGCCGGAGAGATCCGGTGT TCCCTTCGGGGACGTGCACACAGGTGCTGCATGGCTGT CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC AACGAGCGCAACCCACGTCCTCAGTTGCCATCATTGAG TTGGGCACTCTGGGGAGACTGCCGGTGATAAGCCGCG AGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTTA CGGGATGGGCTACACACGTGCTACAATGGCGGTGACA ATGGGCAGCGAAGGGGCGACCTGGAGCGAATCCCCAA AAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGGGT GCATGAAGGCGGAATCG
PPFM16	GGTTCGGAATAACTCAGGGAAACTTGAGCTAATACCG GATACGCCCTTTTGGGGAAAGGTTTACTGCCGGAAGAT CGGCCCGCGTCTGATTAGCTAGTTGGTGGGGTAACGGC

	CTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGAT GATCAGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAA GGCCTTAGGGTTGTAAAGCTCTTTTATCCGGGACGATA ATGACGGTACCGGAGGAATAAGCCCCGGCTAACTTCGT GCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTG CTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCG TTTTAAGTTGGGGGTGAAAGCCTGTGGCTCAACCACAG AATTGCCTTCGATACTGGGACGCTTGAGTGTGGTAGAG GTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAG ATATTCGCAAGAACACCGGTGGCGAAGGCGGCCAACT GGACCATCACTGACGCTGAGGCGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA ACGATGAATGCCAGCCGTTGGGGGGCTTGCCCTTCAGT GGCGCAGCTAACGCTTTGAGCATTCCGCCTGGGGAGTA CGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGG CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC AACGCGCAGAACCTTACCATCCTTTGACATGGCAGGCT AACCAGAGAGATTTGGTGTTCCCTTCGGGGACCTGCAC ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGTC CTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGAC TGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACGT CAAGTCCTCATGGCCCTTACGGGATGGGCTACACACGT GCTACAATGGCGGTGACAGTGGGATGCGAGCCTGCGA AGGTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATT GCACTCTGCAACTCGGGTGCATGAAGGC
PPFM19	GGCAGACGGGTGAGTAACACGTGGGAACGTGCCCTTC GGTTCGGAATAACTCAGGGAAACTTGAGCTAATACCG GATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGAT CGGCCCGCGTCTGATTAGCTTGTTGGTGGGGTAACGGC CTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGAT GATCAGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAA GGCCTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATA ATGACGGTACCGGAAGAATAAGCCCCGGCTAACTTCGT GCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTG

	CTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCC GATTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACA GAATTGCCTTCGATACTGGTTGGCTTGAGACCGGAAGA GGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTA GATATTCGCAAGAACACCAGTGGCGAAGGCGGCTGTC TGGTCCGGTTCTGACGCTGAGGCGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA AACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAG TGGCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGT ACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGG GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG CAACGCGCAGAACCTTACCATCCCTTGACATGGCATGT TACCTCGAGAGATCGGGGATCCTCTTCGGAGGCGTGCA CACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGT CCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGA CTGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACG TCAAGTCCTCATGGCCCTTACGGGATGGGCTACACACG TGCTACAATGGCGGTGACAGTGGGACGCGAAACCGCG AGGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGAT TGCACTCTGCAACTCGGGTGCATGAAGGCGGAATCGCT
PPFM22	GGCAGACGGGTGAGTAACACGTGGGAACGTGCCCTTC GGTTCGGAATAACTCAGGGAAACTTGAGCTAATACCG GATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGAT CGGCCCGCGTCTGATTAGCTTGTTGGTGGGGTAACGGC CTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGAT GATCAGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGG CGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAA GGCCTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATA ATGACGGTACCGGAAGAATAAGCCCCGGCTAACTTCGT GCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTG CTCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCC GATTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACA GAATTGCCTTCGATACTGGTTGGCTTGAGACCGGAAGA GGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTA GATATTCGCAAGAACACCAGTGGCGAAGGCGGCTGTC TGGTCCGGTTCTGACGCTGAGGCGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA

	AACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAG TGGCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGT ACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGG GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG CAACGCGCAGAACCTTACCATCCCTTGACATGGCATGT TACCTCGAGAGATCGGGGATCCTCTTCGGAGGCGTGCA CACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGT CCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGA CTGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACG TCAAGTCCTCATGGCCCTTACGGGATGGGCTACACACG TGCTACAATGGCGGTGACAGTGGGACGCGAAACCGCG AGGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGAT TGCACTCTGCAACTCGGGTGCATGAAGGCGGAATCGCT AGTAAT
PPFM35	GCAGACGGGTGAGTAACACGTGGGAACGTGCCCTTCG GTTCGGAATAACTCAGGGAAACTTGAGCTAATACCGG ATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGATC GGCCCGCGTCTGATTAGCTTGTTGGTGGGGTAACGGCC TACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATG ATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG CAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGG CCTTAGGGTTGTAAAGCTCTTTTGTCCGGGACGATAAT GACGGTACCGGAAGAATAAGCCCCGGCTAACTTCGTG CCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGC TCGGAATCACTGGGCGTAAAGGGCGCGTAGGCGGCCG ATTAAGTCGGGGGTGAAAGCCTGTGGCTCAACCACAG AATTGCCTTCGATACTGGTTGGCTTGAGACCGGAAGAG GACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAG ATATTCGCAAGAACACCAGTGGCGAAGGCGGCTGTCT GGTCCGGTTCTGACGCTGAGGCGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA ACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGT GGCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTA CGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGG CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC AACGCGCAGAACCTTACCATCCCTTGACATGGCATGTT ACCTCGAGAGATCGGGGATCCTCTTCGGAGGCGTGCAC

	ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAG ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGTC CTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGAC TGCCGGTGATAAGCCGCGAGGAAGGTGTGGATGACGT CAAGTCCTCATGGCCCTTACGGGATGGGCTACACACGT GCTACAATGGCGGTGACAGTGGGACGCGAAACCGCGA GGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATT GCACTCTGCAACTCGGGTGCATGAAGGCGGAATCGCTA GTAA

DISCUSSION

5. DISCUSSION

Rice is an important food grain and is a staple food for majority of the world's population. To meet increasing global demand and consumption, rice productivity must be enhanced. The agricultural production over the past few decades became more and more dependent on agrochemicals, as a reliable method of crop protection with economic stability. Increased use of chemical inputs causes several negative effects. It diminishes crop yield, destroys the soil nutrient balance, leads to poor soil quality in terms of physiochemical properties, and gives rise to a variety of plant diseases Thus, a biological substitute for chemical fertilizers that will efficiently promote the plant growth and prevent the depletion of soil fertility and soil quality is essential.

To minimize the use of chemical fertilizers and provide nutrients through other means emphasis is now focused on growing paddy using organic manures such as Farm Yard Manure (FYM) and biofertilizers like *Azotobacter, Azospirillum*, Phoshorous Solubilising Bacteria (PSB), Plant Growth Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhiza (AM fungi) which are ideal measures to produce roots of higher quality and safe. Among these various efficient biofertilizers, methylotrophs employ multiple mechanisms to promote plant growth, which makes them a suitable and promising candidate for use in sustainable agriculture.

Many reports suggest that PPFMs can act as potential agents as plant growth promoters and also help in protecting plants from pathogenic attack. The efficiency of *Methylobacterium* in plant growth promotion could be better exploited and thus has attracted increasing interest in recent years. Many studies proved the positive effect of PPFM on growth and yield of plants. These effects might be mediated by the production or synthesis of plant hormones. They have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins. Several aspects of plant growth promotion by *Methylobacterium* have already been investigated, such as the production of urease enzyme (Holland and Polacco,

1992), stimulation of seed germination and promotion of root growth and morphology (Holland, 1997) and induced systemic resistance (Madhaiyan *et al.*, 2004). Methylotrophs are known to possess antagonistic activities, which can be used to protect plants from pathogens and therefore, to improve the health of plants. Inhibition of phytopathogens by PPFM isolates and other methylotrophs has already been reported (Poorniammal *et al.*, 2009). In the present study, an attempt was made to isolate and characterize Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy.

Several authors have already reported the natural association of PPFMs with plants. It was for the first time, Basile *et al.* (1969) reported these organisms as a contaminant of tissue cultures of the leafy liverwort, *Scapania nemorosa* which lead to the conclusion that these organisms are inhabitants of plant surface. The association of *Methylobacterium* species with plants seems to rely on a symbiotic relationship between the bacterium and host plants. Most common niche for synergism between *Methylobacterium* and plant is the phyllosphere, where they utilize methanol evolved from leaves as the sole source of carbon and energy (Trotsenko *et al.*, 2001).

In the present investigation, PPFMs were isolated from phyllosphere of paddy grown in different agro climatic conditions of Kerala. Isolations were made following leaf imprint method using AMS medium which is a selective medium for isolating PPFM (Lindstrom and Chistoserdova, 2002). In all, 46 methylotrophs were obtained and the isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as the reference culture.

Methylobacterium is able to produce IAA, suggesting that inoculation of this bacteria could increase plant IAA concentrations and promote plant growth (Lee *et al.*, 2006). The presence of IAA was reported in supernatants of PPFM cultures (Omer *et al.*, 2004). There are numerous reports available on indole-3-acetic acid (IAA) production by PPFMs (Omer *et al.*, 2004; Anitha, 2010).

In the present study, all the 47 PPFM isolates were found to produce IAA under *in vitro* conditions. However, it showed wide variations ranging from 9.27 to 68.65 $\mu g\ mL^{-1}$ of culture filtrate.

The first report on the production of IAA in significant amount by methylotrophs was given by Ivanova *et al.* (2001) who detected various indole compounds in the culture liquids of 37 methylotrophic bacteria belonging to different taxa and different strains of *Methylobacterium.* Auxins produced by theses strains were found to range between 3-100 μg mL^{-1}. Omer *et al.,* (2004) unambiguously confirmed by high performance liquid chromatography in combination with nuclear magnetic resonance chromatography (NMR) that PPFM produced plant hormone IAA. Thangamani and Sundaram (2005) and Radha (2007) have documented production of IAA by PPFM ranging from 3.44 μg mL^{-1} to 25.51 μg mL^{-1} and 9.04 μg mL^{-1} to 28.15 μg mL^{-1} respectively.

Carotenoid is an organic pigment that is found widely in plants and microorganisms. They are essential to plants for photosynthesis and protection against destructive photo oxidation (Goodwin, 1984). In photosynthetic bacteria, carotenoid help in harvesting and transferring light energy to chlorophyll and also protect the photosynthetic apparatus against photo oxidation. The widespread occurrence of carotenoid in non-phototrophic bacteria suggests that their presence is crucial for the viability of these organisms in their natural environment and serve as an important taxonomic marker for the identification of isolates. The distinctive pink pigmentation of PPFMs is due to carotenoid, which render them to be tolerant to extreme light condition and radiation.

In the present investigation, all 47 PPFM isolates produced carotenoid pigment in variable quantities ranging from 0.07 to 1.74 μg mL^{-1}. Our results are consistent with the results of Zhohu *et al.*, (2014) who studied the effect of light intensity on the carotenoid pigment production by the photosynthetic bacteria and recorded a carotenoid pigment production of 1.455 μg mL^{-1} at optimum light intensity.

The genus *Methylobacterium* is the commonly noted leaf epiphyte and represents an abundant and stable member of the phyllosphere community of a wide range of crop plants (Hirano and Upper, 1991; Holland and Polacco, 1994 ; Wellner *et al.*, 2011). A positive role is played by phyllosphere antagonistic

microorganisms, which protect the plants from pathogenic microorganisms and thus improve their healthiness (Patkowska, 2003). Methylotrophs are known to possess antagonistic activities, which can be used to protect plants from pathogens and therefore, to improve the health of plants. Inhibition of phytopathogens by PPFM isolates and other methylotrophs has already been reported (Poorniammal *et al*., 2009). Seed treatment or foliar spray of *Methylobacterium* on rice induced the pathogenesis related proteins which protected the plant against sheath blight pathogen *Rhizoctonia solani* under pot culture conditions. Thus the result revealed that, application of *Methylobacterium* reduces the disease incidence on rice (Madhaiyan *et al.,* 2004). Studies on biocontrol potential of *Methylobacterium* has gained considerable attention which is emerging as a promising alternative to chemical control strategies.

In the present investigation, the *in vitro* study revealed that PPFM isolates can inhibit mycelial growth of the fungal pathogens *Rhizoctonia solani* and *Pyricularia oryzae.* These isolates also inhibited the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae.*

Among all the isolates tested, six isolates inhibited *Rhizoctonia solani.* The isolate PPFM10 significantly reduced the linear mycelial growth of *Rhizoctonia solani* with an inhibition zone of 12.72 mm. The reference culture showed antagonistic activity against *R. solani* with a ZOI of 9.07mm diameter. These results are in agreement with the studies of Poorniammal *et al.,* (2010) who reported that *Methylobacterium* isolate CO 47 significantly reduced the linear mycelial growth of *Rhizoctonia solani* to an extent of 52.2 per cent over control with an inhibition zone of 14.00 mm. Four isolates showed antagonistic activity against *Pyricularia oryzae.* PPFM24 exhibited the maximum ZOI of 10.00 mm. Four out of forty seven isolates tested, inhibited *Xanthomonas oryzae (pv) oryzae*. PPFM5 produced the maximum ZOI of 9.80 mm diameter.

PPFMs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins. Seeds treated with the methylotrophic strains improved seed germination, seedling vigor index (SVI) and biomass of rice seedlings. The

methylotrophic population in the treated seedlings increased in the vegetative stages when compared to seeding stages. Treated seedlings showed a higher accumulation of plant hormones viz trans-zeatin riboside, isopentenyladenosine, and indole-3-acetic acid than untreated seedlings (Lee *et al.*, 2006).

Based on these findings, effects of PPFM isolates on paddy seed germination and seedling growth has been tested and the results revealed that the germination percentage of inoculated seeds showed a significant increase compared to uninoculated control. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35. This treatment was found to be significantly superior to the uninoculated control which recorded a germination percentage of 86 per cent and it was 16.28 per cent increase in germination over uninoculated control.

Eight *Methylobacterium* isolates were tested for their effect on seed germination. The isolates, PPFM-SOY (isolated from soybean leaf) and GN (isolated from groundnut leaf) increased the germination percentage of heat-treated seeds of soybean, maize and paddy. When the heated seeds of soybean was treated with PPFM-SOY, 14.28 per cent increase in germination was obtained compared to untreated heated seeds. Same level of increase in germination was observed on treatment with PPFM-GN. When normal seeds were treated with PPFM-SOY and PPFM-GN, 23.21 and 7.14 per cent increase in germination was observed respectively. Treatment of heated maize seeds with PPFM-SOY and PPFM-GN resulted in an increase of 27.50% and 30.0% over control respectively. For paddy seeds also 13.88% and 11.11% increase over control was recorded in treatments with PPFM-SOY and PPFM-GN respectively (Anitha, 2010).

In the present study, root shoot ratio of seedlings showed significant increase when seeds were treated with PPFM isolates. Maximum root shoot ratio of 0.62 was observed when seeds were treated with PPFM26 and PPFM35. These treatments showed 87.88 per cent increase in root shoot ratio over uninoculated control.

The present investigation conclusively proved that, PPFM inoculation in paddy has significant effect on growth parameters like plant height, tiller production and leaf area index as compared to uninoculated plants.

It was interesting to note that application of PPFM isolates significantly influenced the yield and yield attributes of paddy. The per cent increase in yield due to application with PPFM11 was 37.59 against uninoculated control and 20.57 against the reference strain. The beneficial effect of PPFM on crop growth and yield has already been reported in rice (Senthilkumar, *et al.*, 2002). Similar results has been reported in several crops including cotton (Madhaiyan *et al.*, 2005), groundnut (Reddy, 2002), tomato (Thangamani and Sundaram, 2005), soybean, blackgram and sugarcane (Madhaiyan *et al.*, 2005).

Combined inoculation of PPFMs and *Rhizobium* on groundnut cultivar Co(Gn)4 gave significant increase in plant growth, biomass production and yield parameters of groundnut (Reddy *et al.*, 2002). The superior performance of *Coleus forskohlii* inoculated with PPFM isolates may be due to the increased plant growth parameters, resulting in improved biomass and tuber yield.

Several workers reported growth promotional ability of PPFMs in several crops including cotton (Madhaiyan *et al.*, 2005), rice (Senthilkumar, 2003), groundnut (Reddy *et al.*, 2002), tomato (Thangamani and Sundaram, 2005), soybean, blackgram and sugarcane (Madhaiyan *et. al.*, 2005). PPFM inoculation was found to increase the photosynthetic activity by enhancing the number of stomata, chlorophyll concentration and malic acid content of crops (Cervantes-Martinez *et al.*, 2004).

Madhaiyan *et al.,* (2004) observed higher photosynthetic activity in rice cultivar Co-47 that received *Methylobacterium* and attributed the effect due to enhancement of chlorophyll concentration, maleic acid content and increased number of stomata.

The present investigation could establish that, PPFM inoculation to paddy results in significantly higher chlorophyll content as compared to uninoculated plants. At 60 DAT, the maximum chlorophyll content of 1.97 $\mu g\ gram^{-1}$ of sample

tissue was recorded in plants treated with PPFM46 compared to control (1.40 µg g^{-1}).

Proline is one of the most important osmolytes that accumulate in plants during severe drought stress (Yoshiba *et al.,* 1997). It not only act as an osmolyte for osmotic adjustment but also helps to stabilize sub-cellular structures (e.g. proteins and membranes). It is also involved in the scavenge of free radicals and buffering cellular redox potential. In the present investigation, proline content of the plants inoculated with PPFM ranged between 5.09 to 285.51 µ moles gram $tissue^{-1}$. Treatment with PPFM19 increased the proline content by 674.77 per cent over the uninoculated control. Also, PPFM treatments had significant influence on plant cell membrane stability index. Significantly higher cell membrane integrity of 80.83% was recorded with PPFM28. The results obtained here are in confirmation with the findings of Sivakumar *et al.,* (2017) who reported treatment of plants with *Methylobacterium spp.* has to lead to an increase in proline levels. Foliar application of PPFM (2%) increased the proline content by 11.34 per cent in tomato compared to absolute control under stressed condition. Here the treated plant recorded 326.45 µg g^{-1} of proline as against 162.66 µg g^{-1} in absolute control.

As none of the isolates showed superior performance for all the parameters evaluated, different parameters such as seed germination percentage, seedling vigour index and grain yield of paddy were given comparative ranks from 3 to 1 based on the importance and weighted average rank was calculated for different treatments. Also, by considering the growth promotion efficacy, the isolates PPFM11, PPFM16, PPFM19, PPFM22 and PPFM35 were adjudged as superior isolates.

In fact all the selected PPFM isolates were performed even better than the reference strain (Figure 1 and 2). These selected isolates significantly improved the seed germination and seedling growth of paddy compared to uninoculated control and reference strain (Figure 3 and 4). Also, these selected isolates significantly enhanced the growth, yield and yield attributes. Effect of these

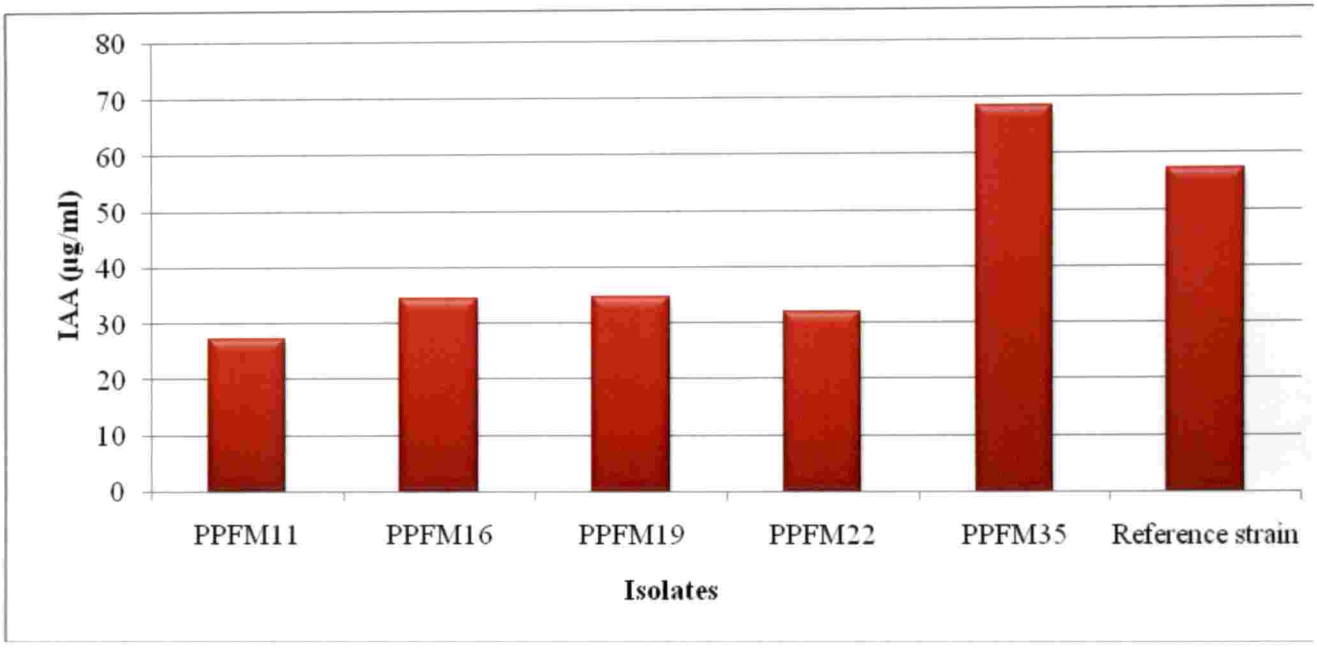


Figure 1. Indole acetic acid production by selected PPFM isolates

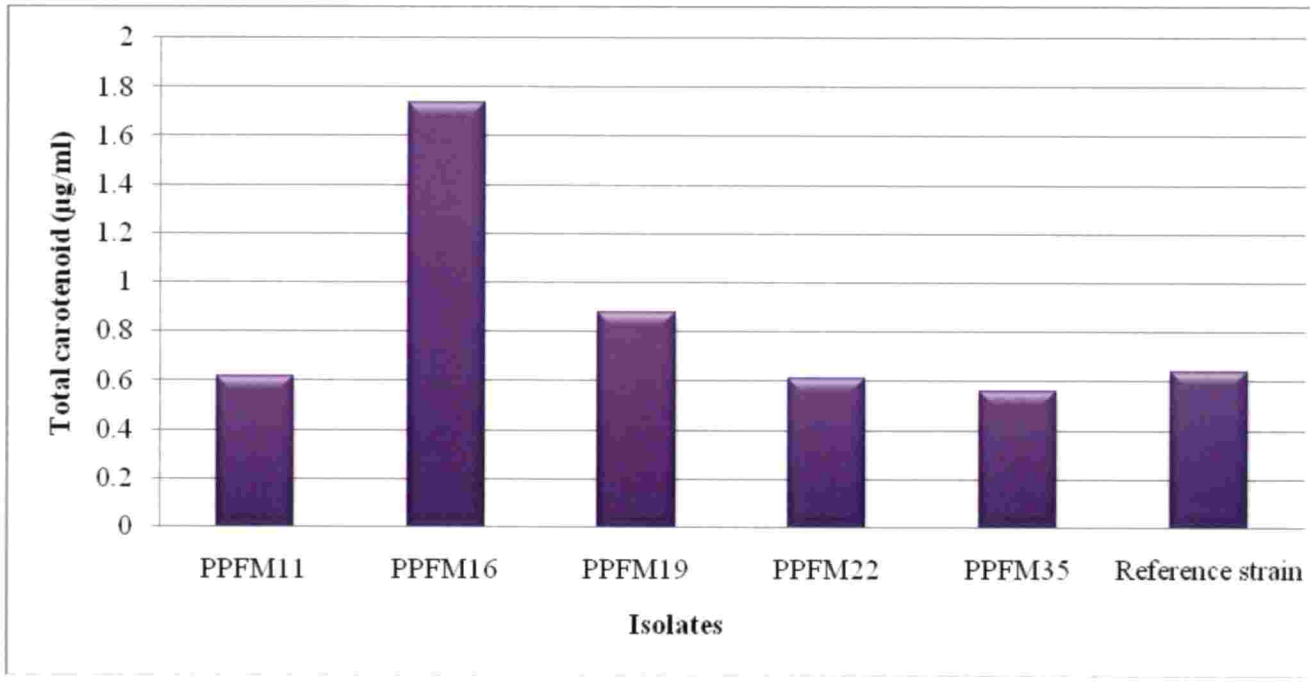


Figure 2. Carotenoid pigment production by selected PPFM isolates

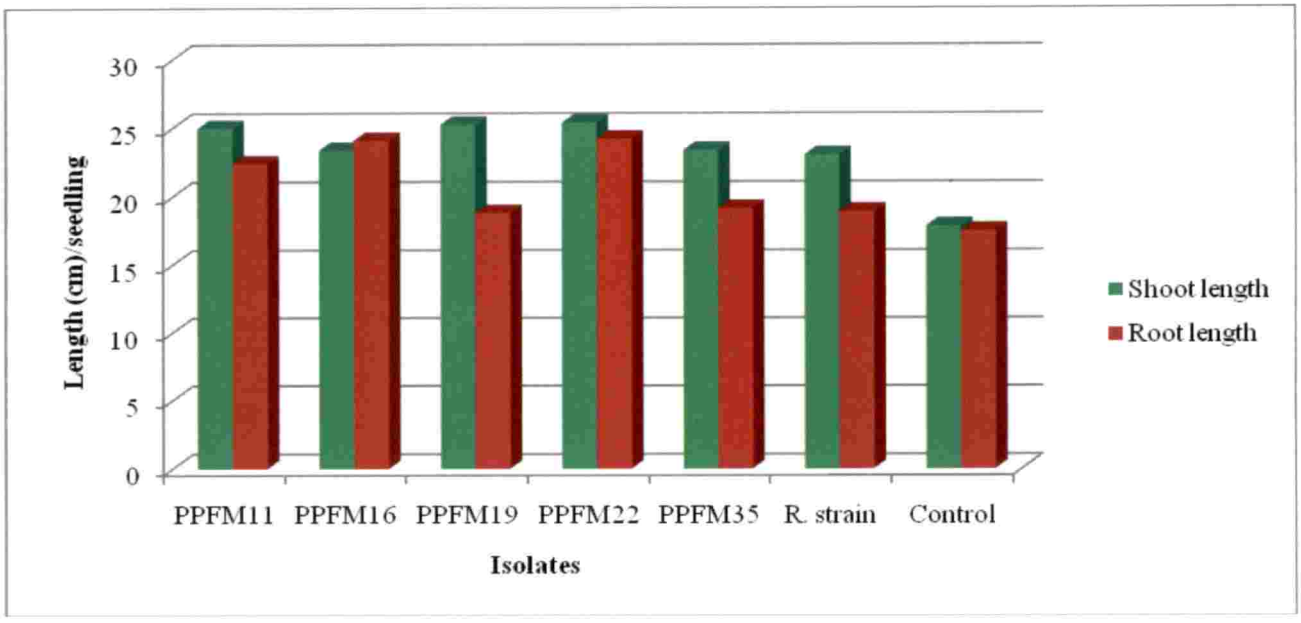


Figure 3. Effect of selected PPFM isolates on shoot and root length of paddy seedlings

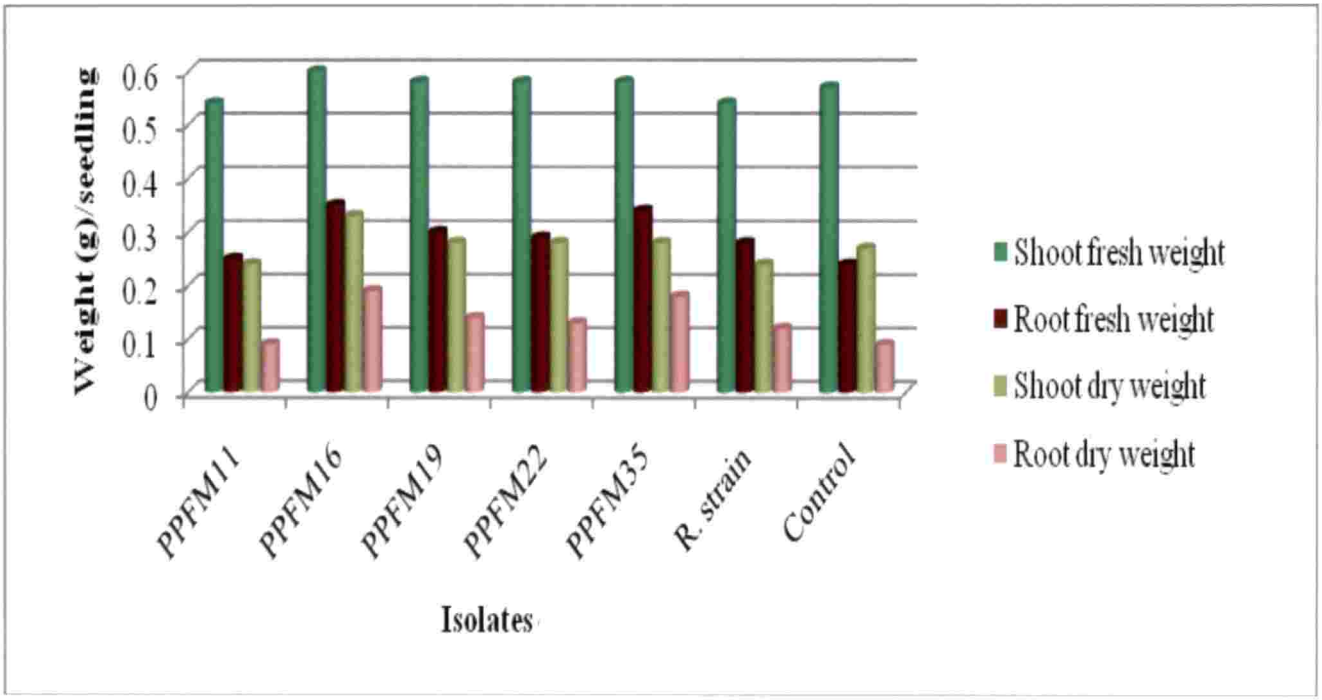


Figure 4. Effect of selected PPFM isolates on fresh and dry weight of shoot and root of paddy seedlings

selected PPFM isolates on dry matter production of paddy is represented in Figure 5.

The per cent increase in seedling root shoot ratio due to inoculation with selected PPFM strains ranged from 9.09 to 87.88 per cent against uninoculated control.

Significant increase in growth parameters was observed in plants treated with selected PPFM strains. They improved plant height and tiller production compared to control plants.

Similarly, the PPFM strains improved the physiological parameters of the plant such as total chlorophyll content, proline content and cell membrane stability index compared to control (Figure 6, 7 and 8). These results confirmed previous studies with increase of rice and sugarcane growth, when these crops were treated with *Methylobacterium* strains (Madhaiyan *et al.*, 2005).

In the present investigation, seeds treated with these selected PPFM isolates recorded 9.30 to16.28 per cent increase in seed germination over control and 1.06 to 6.38 per cent increase over reference strain.

Pattanashetty *et al.* (2012) reported that pink pigmented facultatively methylotrophic (PPFM) bacterium influences seed germination and seedling growth by producing the plant growth regulator zeatin and related cyotkinins and germination of both fresh and aged seed is enhanced by treatment with PPFMs.

The per cent increase in seedling vigour index due to the application of selected PPFM isolates ranged from 37.66 to 56.57 over control. These isolates also had significant effect on seedling vigour index (SVI) compared to reference strain, which recorded 6.05 to 20.61 per cent increase in SVI over reference strain.

Selected PPFM isolates recorded 0.15 to 37.59 per cent yield increase over control and this is in line with the study of Senthilkumar (2003) who reported yield increase in paddy due to PPFM inoculation. Also, these isolates were superior to reference strain with 11.85 to 20.57 per cent increase of grain yield over reference strain.

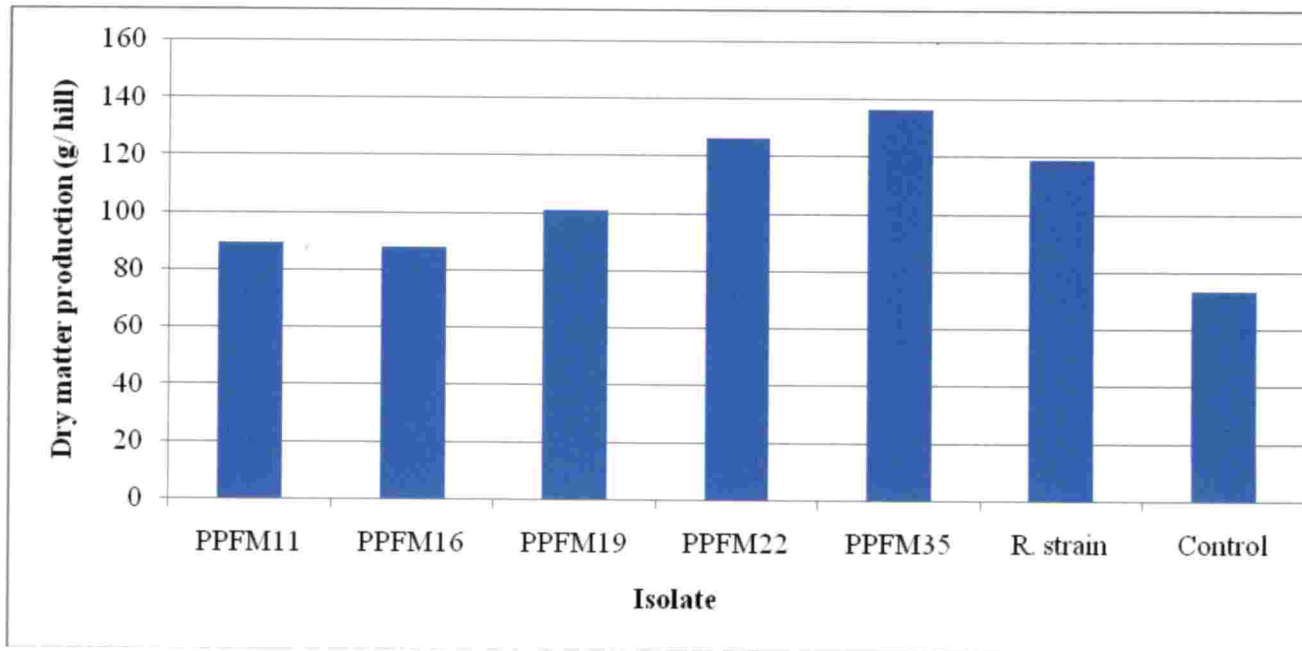


Figure 5. Effect of selected PPFM isolates on dry matter production of paddy

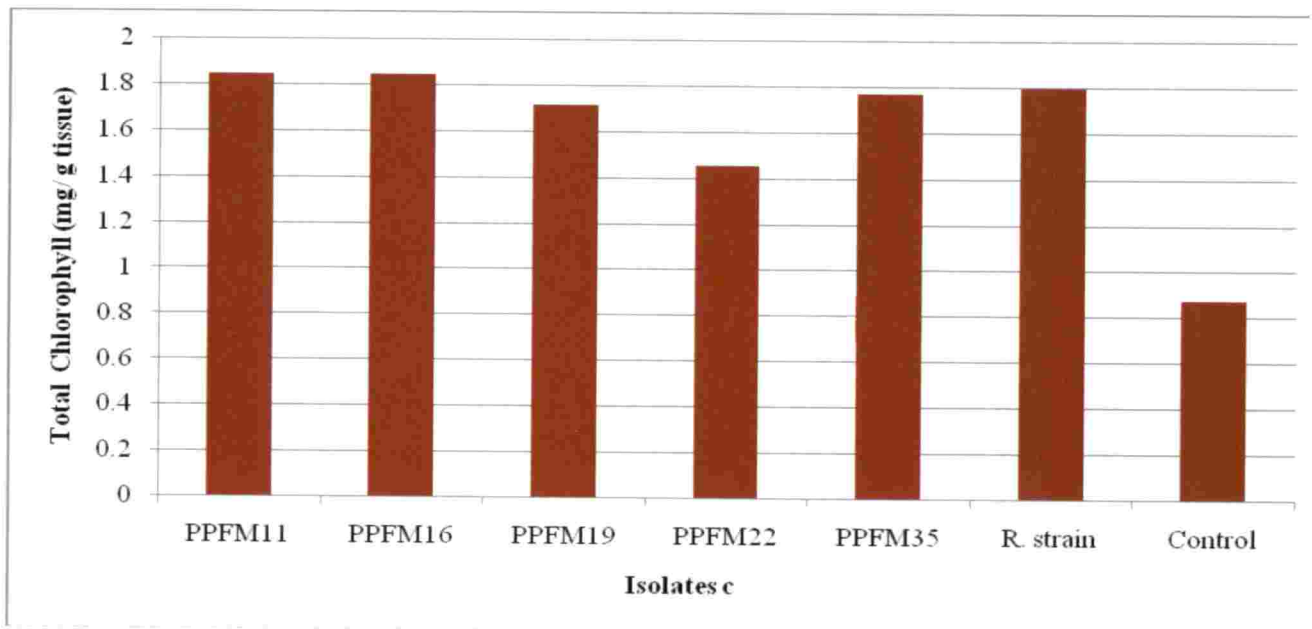


Figure 6. Effect of selected PPFM isolates on chlorophyll content of paddy at 60 days after transplanting

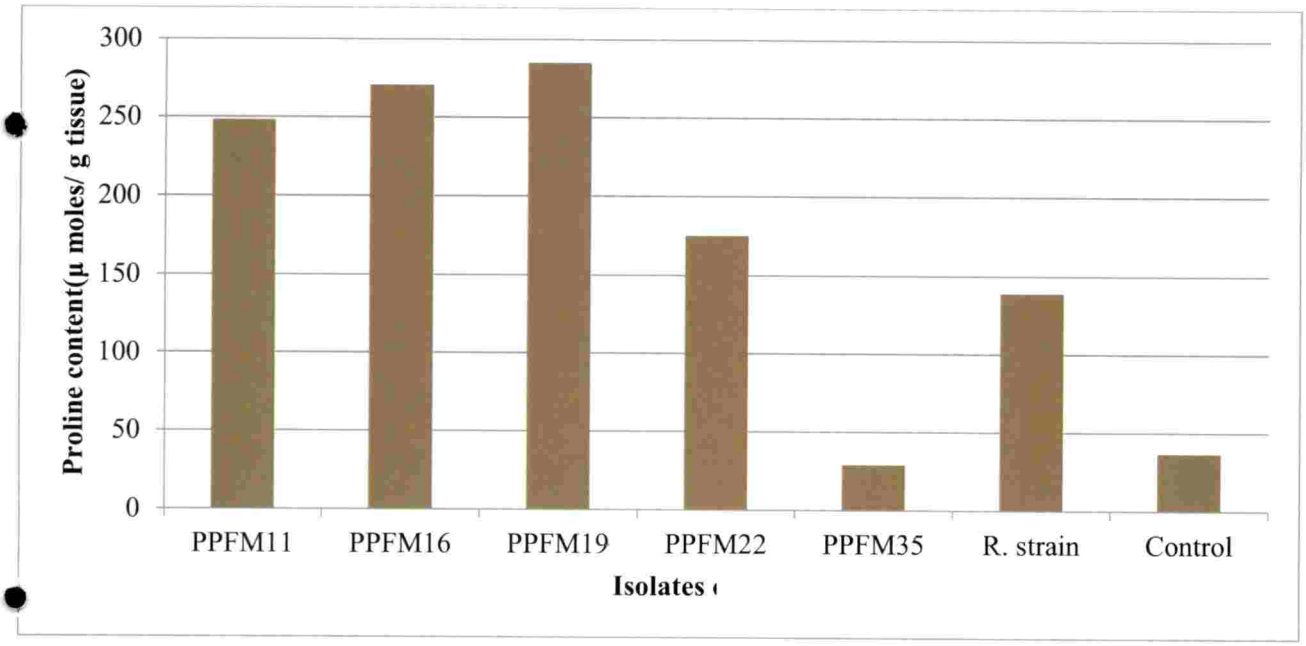


Figure 7. Effect of selected PPFM isolates on proline content of paddy at 60 days after transplanting

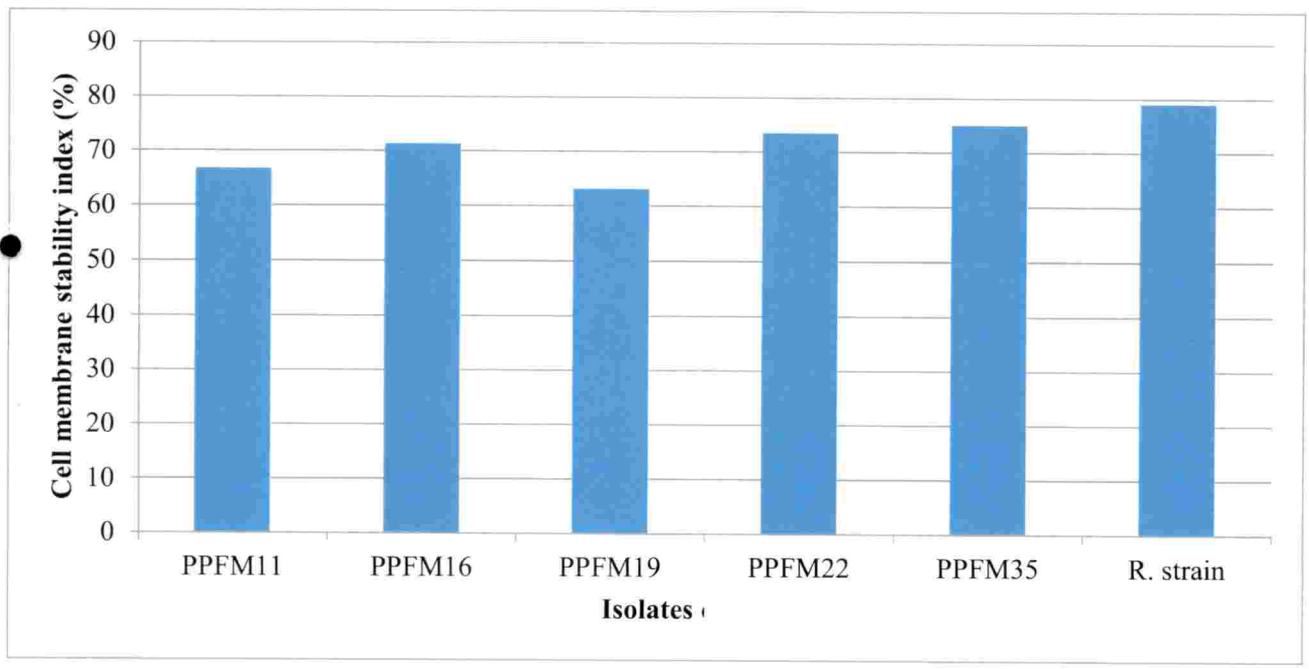


Figure 8. Effect of selected PPFM isolates on cell membrane stability index of paddy at 60 days after transplanting

In the present study, the application of selected PPFM isolates significantly influenced the dry matter production of paddy compared to control plants. The per cent increase in dry matter production of paddy over control by selected PPFM isolates is represented in Figure 9.

There was notable per cent increase in physiological parameters such as total chlorophyll content and proline content of paddy over control when treated with selected PPFM isolates (Figure 10 and 11).

Microscopic studies revealed that all the PPFM isolates were rod shaped, motile, gram negative and produce poly β-hydroxy butyrate granules (Green and Bousifield, 1982). In the present study, the superior isolates selected were subjected to morphological characterization. The results revealed that all the isolates were rod shaped, stained Gram negative and exhibited motility. Further one week after incubation, pink colonies with varying color intensities were observed on AMS medium. The expression of pink pigmentation with varied level of intensity in PPFM indicates the presence of carotenoids (Fasim, 2003) which is known to protect these bacteria from intense light and UV radiation (Liu *et al.*, 1993).

These isolates were further subjected to a series of biochemical tests to study their relationship with the already existing genus *Methylobacterium.* All isolates were aerobes producing catalase and oxidase as already demonstrated by Bellin and Spain (1976) and positive for urease test and indole production (Thangamani, 2005). However, hydrolysis of casein, starch, cellulose degradation, MR and VP test and nitrate reduction test was not recorded in any of the isolates.

The methylotrophic bacteria are capable of growing on single carbon compounds such as formate, formaldehyde and methanol as sole source of carbon and energy. They can utilize wide range of multi carbon growth substrates making them facultatively methylotrophic (Lidstrom, 1992). Based on their carbon utilization pattern, classification of methylotrophic bacteria at the species level has been established by Green and Bousifield (1982). In the present study, all the selected isolates were tested for the utilization of 29 different

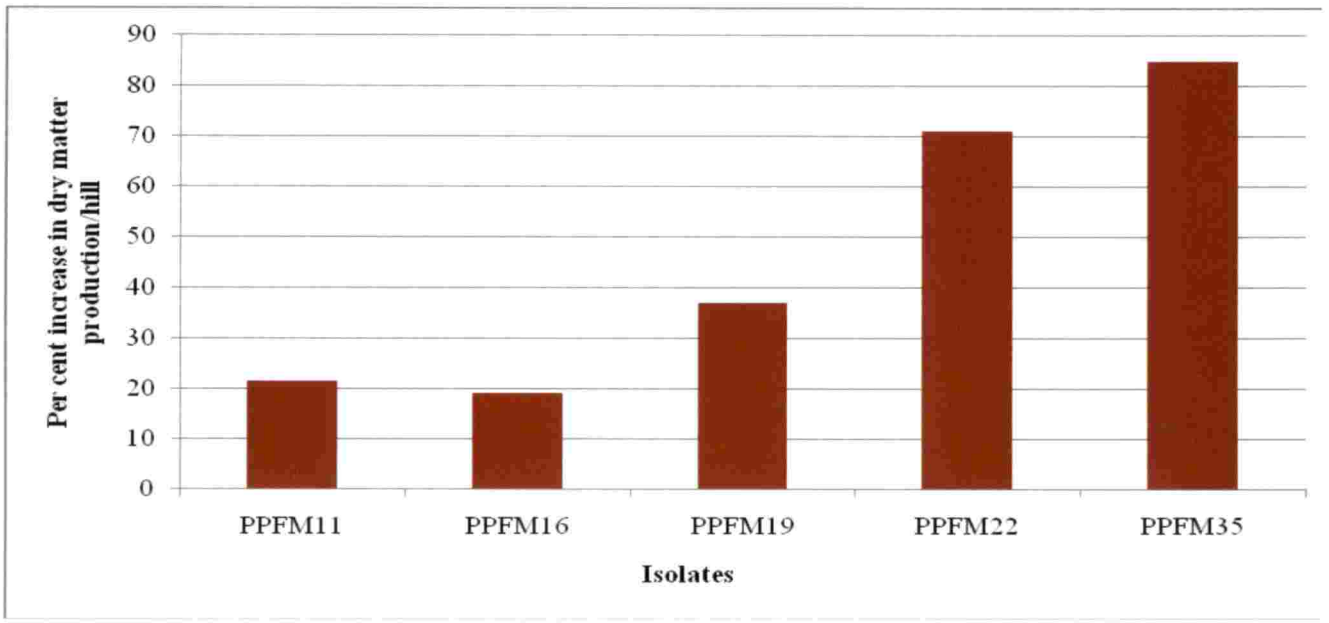


Figure 9. Per cent increase in dry matter production over control by selected PPFM isolates

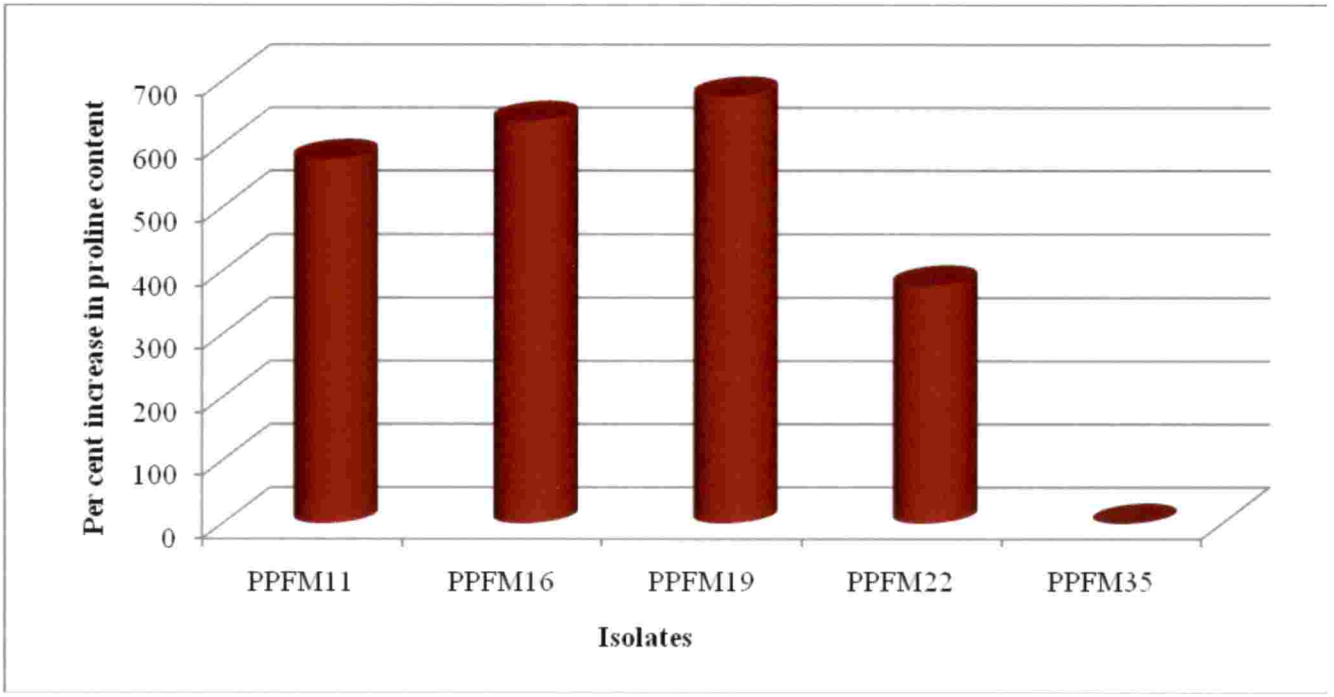
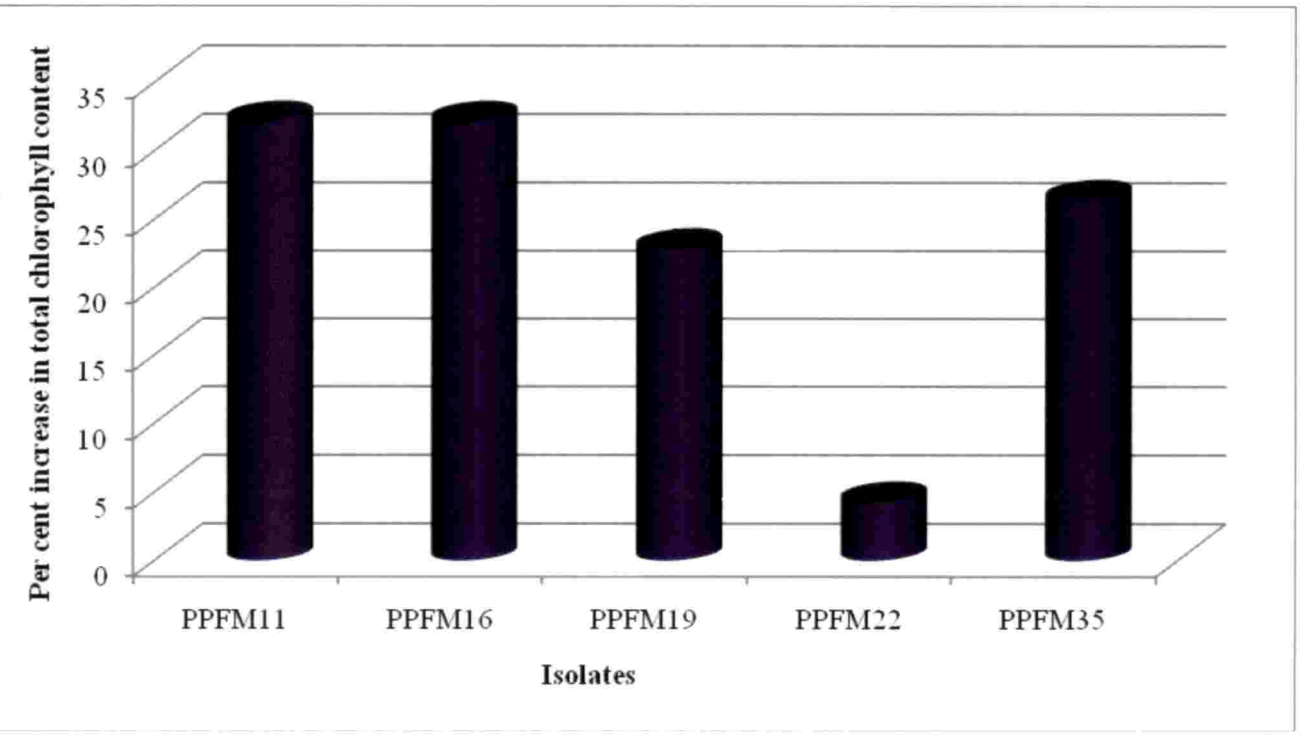


Figure 10. Per cent increase in proline content of paddy over control by selected PPFM isolates



ure 11. Per cent increase in chlorophyll content of paddy over control by selected PPFM isolates

carbon compounds and they showed wide variability in carbon utilization pattern.

The present study demonstrated that it is possible to distinguish and classify the methylotrophic bacteria using 16S rRNA sequence analysis. Our results also indicated that phylogenetic relationships based on 16S rRNA sequences reflect the classical taxonomic classification systems based on phenotypic characteristics for methylotrophs. Thus, 16S rRNA sequence analysis could be a useful tool for detailed classification of methylotrophs. 16S rRNA gene phylogenetic analysis performed clearly showed the position of the isolates within the genus *Methylobacterium*. Isolates PPFM11 and PPFM16 were found to be very close to *M. aquaticum* and *M. radiotolerans* respectively whereas PPFM19, PPFM22 and PPFM35 were found to be very close to *M. populi.*

SUMMARY

6. SUMMARY

Methylobacterium spp. are a group of bacteria known as pink-pigmented facultative methylotrophs (PPFMs) and they are capable of growing on single carbon compounds such as formate, formaldehyde, and methanol as well as on a variety of multicarbon compound having no carbon-carbon bonds. They are distributed ubiquitously in the plant phyllosphere and rhizosphere and have been isolated from many species of plants. Methylotrophs are known to play an important role in increasing crop yield and soil fertility. Their phosphate acquisition, nitrogen fixation, iron chelation and phytohormone production abilities make them promising candidates as biofertilizers. In this context the programme entitled "Isolation and characterization of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy" was undertaken in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram.

The main objectives of the present study were isolation, characterization and evaluation of Pink-Pigmented Facultative Methylotrophs (PPFMs) associated with paddy for antagonistic efficiency, seed germination, seedling growth and yield of paddy. The salient findings of the present study are summarized below.

Forty six isolates were obtained from different locations in Kerala and code numbers were allotted for each of the isolate. They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on Ammonium Mineral Salt (AMS) agar media with methanol as sole source of carbon and energy. The isolate obtained from the commercial product of Tamil Nadu Agricultural University was taken as reference culture.

Indole- Acetic Acid (IAA) production by the different isolates of PPFMs showed wide variations ranging from 9.27 to 68.65 $\mu g\ mL^{-1}$ of culture filtrate.

All the isolates were found to produce carotenoid pigment in varied quantities ranging from 0.07 to 1.74 $\mu g\ mL^{-1}$.

The antagonistic activities of all PPFM isolates were assessed against important fungal pathogens of paddy namely, *Rhizoctonia solani, Pyricularia oryzae* and one bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* following dual culture method. Six out of forty seven isolates tested, inhibited *Rhizoctonia solani.* PPFM10 exhibited the maximum zone of inhibition (ZOI) of 12.72 mm diameter. Among forty seven isolates tested, four isolates inhibited *Xanthomonas oryzae* pv. *oryzae* and PPFM5 produced the maximum ZOI of 9.80 mm diameter. Four isolates showed antagonistic activity against *Pyricularia oryzae* and PPFM24 exhibited the maximum zone of inhibition of 10.00 mm.

Studies on the effect of isolates of PPFMs on paddy seed germination and seedling growth indicated that, both treated seeds and uninoculated control germinated on the second day of seed treatment, but the germination percentage of inoculated seeds showed a significant increase compared to uninoculated control. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35.

Significant increase in seedling shoot length, root length, seedling vigour index, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight and root shoot ratio compared to control, was observed when seeds were treated with PPFM isolates. Inoculation with PPFM30 recorded the maximum shoot length of 26.38 cm and maximum root length of 24.20 cm was obtained in seeds treated with PPFM22 and the same isolate recorded the highest seedling vigour index of 4756.35 over the control (3037.91). The root shoot ratio of seedlings showed significant increase when seeds were treated with PPFM isolates. Maximum root shoot ratio of 0.62 was observed when seeds were treated with PPFM26 and PPFM35 compared to control (0.33).

By assessing the plant growth promotion in paddy, significantly higher values with respect to biometric parameters such as plant height, tiller production and leaf area index were observed in plants treated with PPFM isolates.

Treatments exerted significant effect on the yield and yield attributes of the plant. Inoculation of PPFM significantly increased the yield of with paddy. PPFM11 recording the highest grain yield of 46.30 g $hill^{-1}$ whereas the control recorded a grain yield of 33.65 g $hill^{-1}$ only.

Analysis of the data on physiological parameters of the plants indicated that PPFM isolates were found to have significantly superior in proline content, chlorophyll content and cell membrane stability index over the control.

As none of the treatments showed superior performance for all the parameters evaluated, the five treatments having top weighted average ranks were selected for further characterization. Growth promotion efficacy of the isolates was also considered before finalizing the five efficient isolates. The isolates having top weighted average ranks were PPFM11, PPFM16, PPFM19, PPFM22 and PPFM35.

The superior isolates PPFM11, PPFM16, PPFM19, PPFM22 and PPFM35 were characterized based on morphological, biochemical and molecular characters. Molecular characterization based on 16S rrevealed that the isolates PPFM11 and PPFM16 were found to be very close to *M. aquaticum* and *M. radiotolerans* respectively whereas PPFM19, PPFM22 and PPFM35 were found to be very close to *M. populi.*

In the present investigation, five PPFM isolates were selected as superior isolates. Further studies on the effect of these isolates on plants are required before developing commercial formulations. Hence, the future studies may be focused on the following.

1. Effect of PPFM isolates on crop nutrition such as phosphorous and potassium solubilization and nitrogense activity.

2. Detection and quantification of phytohormones such as cytokinin and gibberellic acid produced by PPFM isolates need to be elucidated.

3. Evaluation of performance of these PPFM isolates on paddy under water stress condition.

4. Evaluation of antagonistic efficiency of the PPFM isolates under pot culture and field condition.

5. Evaluation of synergistic effect of PPFM consortium with different beneficial traits and utilization in upland rice cultivation.

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APPENDICES

APPENDIX - I

COMPOSITION OF MEDIA USED

1. Ammonium Mineral Salt Agar

$(NH_4)_2SO_4$	- 0.5g
K_2HPO_4	- 0.7g
KH_2PO_4	- 0.54g
$MgSO_4.7H_2O$	- 1.0g
$CaCl_2.2H_2O$	- 0.2g
$FeSO_4.7H_2O$	- 4 mg
$ZnSO_4.7H_2O$	- 100µg
$MnCl_2.4H_2O$	- 30 µg
H_3BO_3	- 300 µg
$CoCl_2.6H_2O$	- 200 µg
$CuCl_2.2H_2O$	- 10 µg
$NiCl_2.6H_2O$	- 20 µg
$Na_2MoO_4.2H_2O$	- 60 µg
Agar-agar	- 20g
Distilled water	- 1000 ml

$(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min. After cooling, all other nutrients (sterilised by filtration through a 0.2 µm pore size membrane filter) were added aseptically, followed by 5ml of methanol and 10 µg of cycloheximide were added.

2. Glycerol Peptone Agar

Glycerol	- 10 ml
Peptone	- 10g
Agar-agar	- 20g
Distilled water	- 1000 ml

Glycerol and peptone were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

3. Potato Dextrose Agar

Peeled and sliced potatoes	- 200g
Dextrose ($C_6H_{12}O_6$)	- 20g
Agar-agar	- 20g
Distilled water	- 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 ml of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in to the mixture. The volume was made up to 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 121 °C for 15 min.

3. Potato Sucrose Agar

Peeled and sliced potatoes	- 300g
Peptone	- 5g

Na_2HPO_4	- 2g
$Ca(NO_3)_2$	- 0.5g
Sucrose	- 20g
Agar-agar	- 20g
Distilled water	- 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 ml of distilled water. The potato extract was mixed in the molten agar and Na_2HPO_4, $Ca(NO_3)_2$ and sucrose were dissolved in to the mixture. The volume was made up to 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 121 °C for 15 min.

APPENDIX - II

COMPOSITION OF STAIN USED

1. Crystal violet

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. Gram's iodine

Iodine crystals	- 1.0g
Potassium iodide	- 2.0g
Distilled water	- 300ml

3. Safranin

Ten ml saturated solution of safranin in 100 ml distilled water.

APPENDIX - III

SEQUENCE PRODUCING SIGNIFICANT ALIGNMENTS

a. PPFM11

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured bacterium clone ncd253b08c1 16S ribosomal RNA gene, partial sequence	2165	2165	100%	0.0	99%	HM269657.1
Methylobacterium indicum strain SE2.11 16S ribosomal RNA, partial sequence	2159	2159	100%	0.0	99%	NR_135210.1
Uncultured bacterium partial 16S rRNA gene, clone MD02H10	2154	2154	100%	0.0	99%	FM874456.1
Methylobacterium platani gene for 16S ribosomal RNA, partial sequence, strain: JoN5	2150	2150	100%	0.0	99%	LC025989.1
Methylobacterium sp. 189 partial 16S rRNA gene, strain 189	2150	2150	100%	0.0	99%	FN868943.1
Uncultured bacterium clone ncd897c09c1 16S ribosomal RNA gene, partial sequence	2148	2148	100%	0.0	99%	HM308481.1
Methylobacterium aquaticum DNA, complete genome, strain: MA-22A	2145	21221	100%	0.0	99%	AP014704.1
Methylobacterium aquaticum gene for 16S ribosomal RNA, partial sequence, strain: TH37	2145	2145	100%	0.0	99%	LC026011.1
Methylobacterium platani gene for 16S ribosomal RNA, partial sequence, strain: KB7	2145	2145	100%	0.0	99%	LC025994.1
Methylobacterium platani gene for 16S ribosomal RNA, partial sequence, strain: JiF13	2145	2145	100%	0.0	99%	LC025976.1
Uncultured bacterium clone ncd1094c09c1 16S ribosomal RNA gene, partial sequence	2145	2145	100%	0.0	99%	HM337241.1

b. PPFM16

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium sp. BFE14D 16S ribosomal RNA gene, partial sequence	2111	2111	100%	0.0	99%	KM186992.1
Methylobacterium sp. NG10 16S ribosomal RNA gene, partial sequence	2111	2111	100%	0.0	99%	KC702833.1
Methylobacterium aerolatum strain 5413S-11 16S ribosomal RNA gene, partial sequence	2111	2111	100%	0.0	99%	NR_044130.1
Methylobacterium aerolatum gene for 16S ribosomal RNA, partial sequence, strain: 92a-8	2106	2106	100%	0.0	99%	AB986551.1
Methylobacterium aerolatum gene for 16S ribosomal RNA, partial sequence, strain: 86a	2095	2095	100%	0.0	99%	AB698711.1
Methylobacterium aerolatum strain S8-665 16S ribosomal RNA gene, partial sequence	2089	2089	100%	0.0	99%	JQ660275.1
Methylobacterium sp. NG08 16S ribosomal RNA gene, partial sequence	2084	2084	100%	0.0	99%	KC702831.1
Methylobacterium aerolatum gene for 16S ribosomal RNA, partial sequence, strain: z18b	2073	2073	100%	0.0	99%	AB698677.1
Methylobacterium sp. P53 16S ribosomal RNA gene, partial sequence	2067	2067	100%	0.0	99%	KJ604930.1
Uncultured Methylobacterium sp. clone Cobs2TisG9 16S ribosomal RNA gene, partial sequence	2061	2061	100%	0.0	99%	EU246828.1
Methylobacterium persicinum strain 002-165 16S ribosomal RNA gene, partial sequence	2039	2039	100%	0.0	99%	NR_041442.1
Methylobacterium sp. PB138 gene for 16S rRNA, partial sequence, strain: PB138	2039	2039	100%	0.0	99%	AB220085.1
Methylobacterium fujisawaense 16S ribosomal RNA gene, partial sequence	2028	2028	100%	0.0	98%	AY169421.1
Methylobacterium komagatae strain KPE62102H 16S ribosomal RNA, partial sequence	1995	1995	100%	0.0	98%	HQ009873.1
Methylobacterium komagatae gene for 16S ribosomal RNA, partial sequence, strain: 37e	1978	1978	100%	0.0	98%	AB698710.1
Methylobacterium sp. Ku1409-1-17 gene for 16S rRNA, partial sequence	1956	1956	100%	0.0	97%	LC114094.1
Methylobacterium radiotolerans strain VRI6-3 16S ribosomal RNA gene, partial sequence	1951	1951	100%	0.0	97%	KY882067.1

c. PPFM19

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium thiocyanatum strain DSM 11490 16S ribosomal RNA gene, partial sequence	2215	2215	100%	0.0	99%	NR_112237.1
Methylobacterium populi strain BJ001 16S ribosomal RNA, partial sequence	2207	2207	100%	0.0	99%	NR_074257.1
Methylobacterium populi strain BJ001 16S ribosomal RNA, partial sequence	2207	2207	100%	0.0	99%	NR_029082.1
Methylobacterium aminovorans strain JCM 8240 16S ribosomal RNA gene, partial sequence	2207	2207	100%	0.0	99%	NR_041025.1
Methylobacterium suomiense strain NCIMB 13778 16S ribosomal RNA gene, partial sequence	2196	2196	100%	0.0	99%	NR_041030.1
Methylobacterium rhodesianum strain DSM 5687 16S ribosomal RNA gene, partial sequence	2196	2196	100%	0.0	99%	NR_041028.1
Methylobacterium thiocyanatum strain ALL/SCN-P 16S ribosomal RNA, partial sequence	2191	2191	100%	0.0	99%	NR_044792.1
Methylobacterium zatmanii strain DSM 5688 16S ribosomal RNA gene, partial sequence	2191	2191	100%	0.0	99%	NR_041031.1
Methylobacterium extorquens strain IAM 12631 16S ribosomal RNA gene, partial sequence	2191	2191	100%	0.0	99%	NR_112230.1
Methylobacterium extorquens strain TK 0001 16S ribosomal RNA, partial sequence	2191	2191	100%	0.0	99%	NR_025856.1
Methylobacterium pseudosasae strain BL44 16S ribosomal RNA, partial sequence	2182	2182	100%	0.0	99%	NR_108240.1
Methylobacterium lusitanum strain NCIMB 13779 16S ribosomal RNA gene, partial sequence	2180	2180	100%	0.0	99%	NR_112233.1

d. PPFM22

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium populi BJ001, complete genome	2159	10771	100%	0.0	100%	NC_010725.1
Methylobacterium aquaticum DNA, complete genome, strain: MA-22A	1882	18551	100%	0.0	96%	NZ_AP014704.1
Methylobacterium gossipiicola strain Gh-105, whole genome shotgun sequence	1866	1866	100%	0.0	95%	NZ_FOPM01000066.1
Methylobacterium aquaticum plasmid pMaq22A_1p DNA, complete genome, strain: MA-22A	1816	1816	100%	0.0	95%	NZ_AP014705.1
Methylobacterium nodulans ORS 2060, complete genome	1810	12675	100%	0.0	95%	NC_011894.1
Methylobacterium radiotolerans JCM 2831, complete genome	1810	7232	100%	0.0	95%	NC_010505.1
Methylobacterium radiotolerans JCM 2831 plasmid pMRAD01, complete sequence	1805	3610	100%	0.0	95%	NC_010510.1
Methylobacterium phyllostachyos strain BL47, whole genome shotgun sequence	1794	1794	100%	0.0	94%	NZ_FNHS01000043.1
Microvirga flocculans ATCC BAA-817 L879DRAFT_scaffold00029.29_C, whole genome shotgun sequence	1720	1720	100%	0.0	93%	NZ_JAEA01000030.1
Microvirga guangxiensis strain CGMCC 1.7666, whole genome shotgun sequence	1720	1720	100%	0.0	93%	NZ_FMVJ01000032.1
Microvirga vignae strain BR3299 T20BR3299_1__paired__contig_82, whole genome shotgun sequence	1720	1720	100%	0.0	93%	NZ_LCYG01000082.1

e. PPFM35

Descriptions

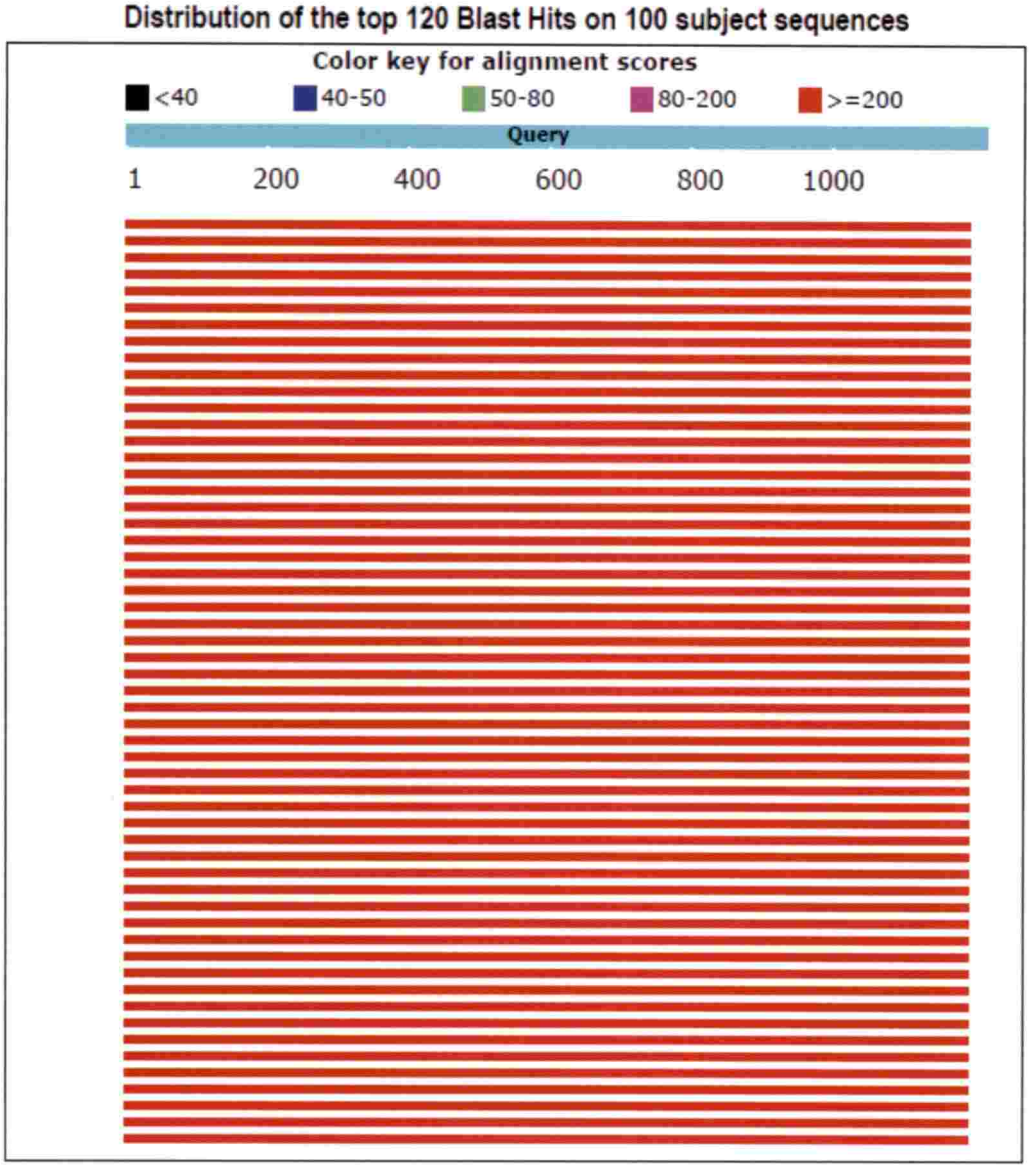
Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Methylobacterium populi strain MSSRF_1B39 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	MG597196.1
Methylobacterium populi strain M2-1 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882116.1
Methylobacterium populi strain M1-2 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882115.1
Methylobacterium populi strain ICGV-1 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882108.1
Methylobacterium populi strain CO6-3 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882101.1
Methylobacterium populi strain N3-3 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882097.1
Methylobacterium populi strain VRI7-1 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882071.1
Methylobacterium populi strain VRI2-A3 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882063.1
Methylobacterium populi strain VRI2-3 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882059.1
Methylobacterium populi strain TMV7-4 16S ribosomal RNA gene, partial sequence	2226	2226	100%	0.0	100%	KY882050.1

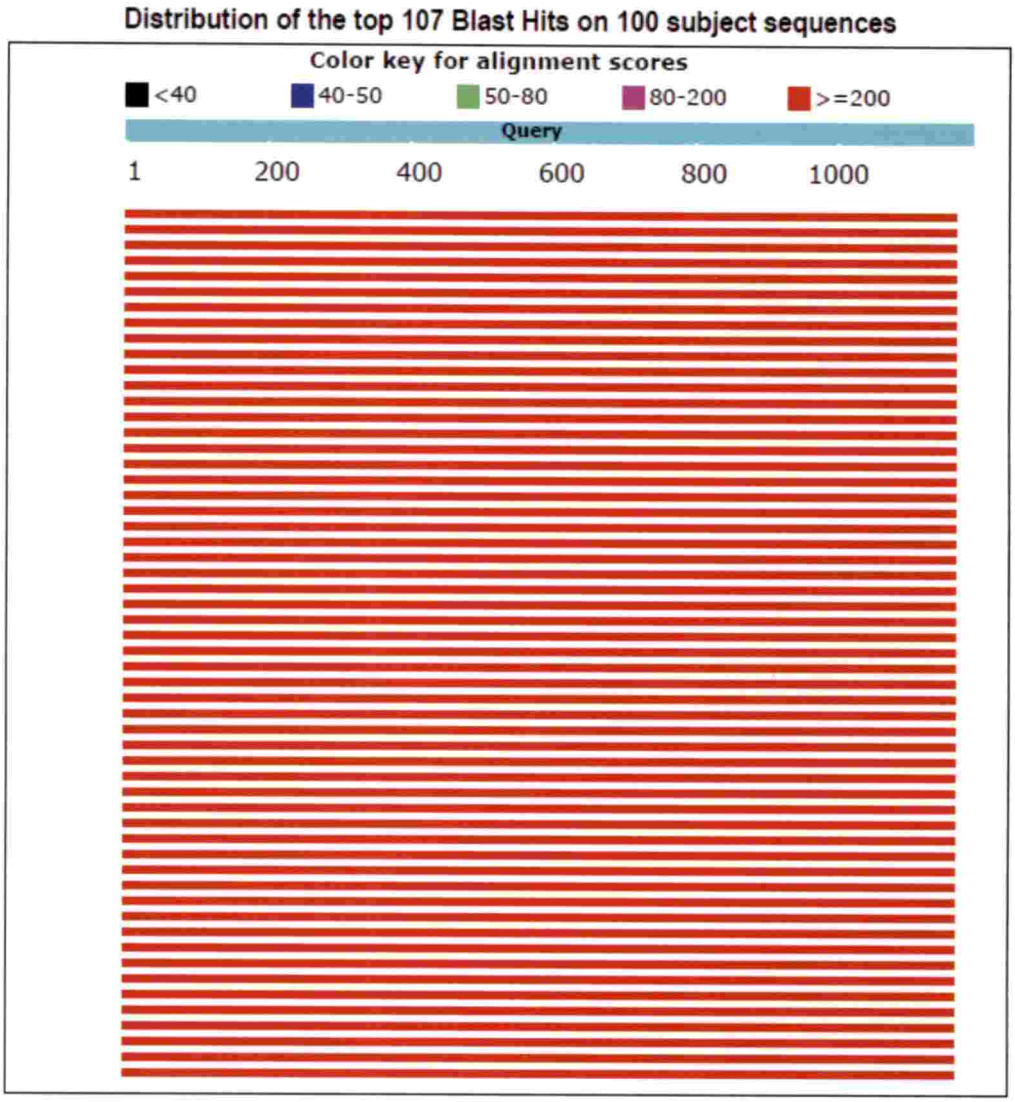
APPENDIX – IV

DISTRIBUTION OF TOP BLAST HITS ON 100 SUBJECT SEQUENCES

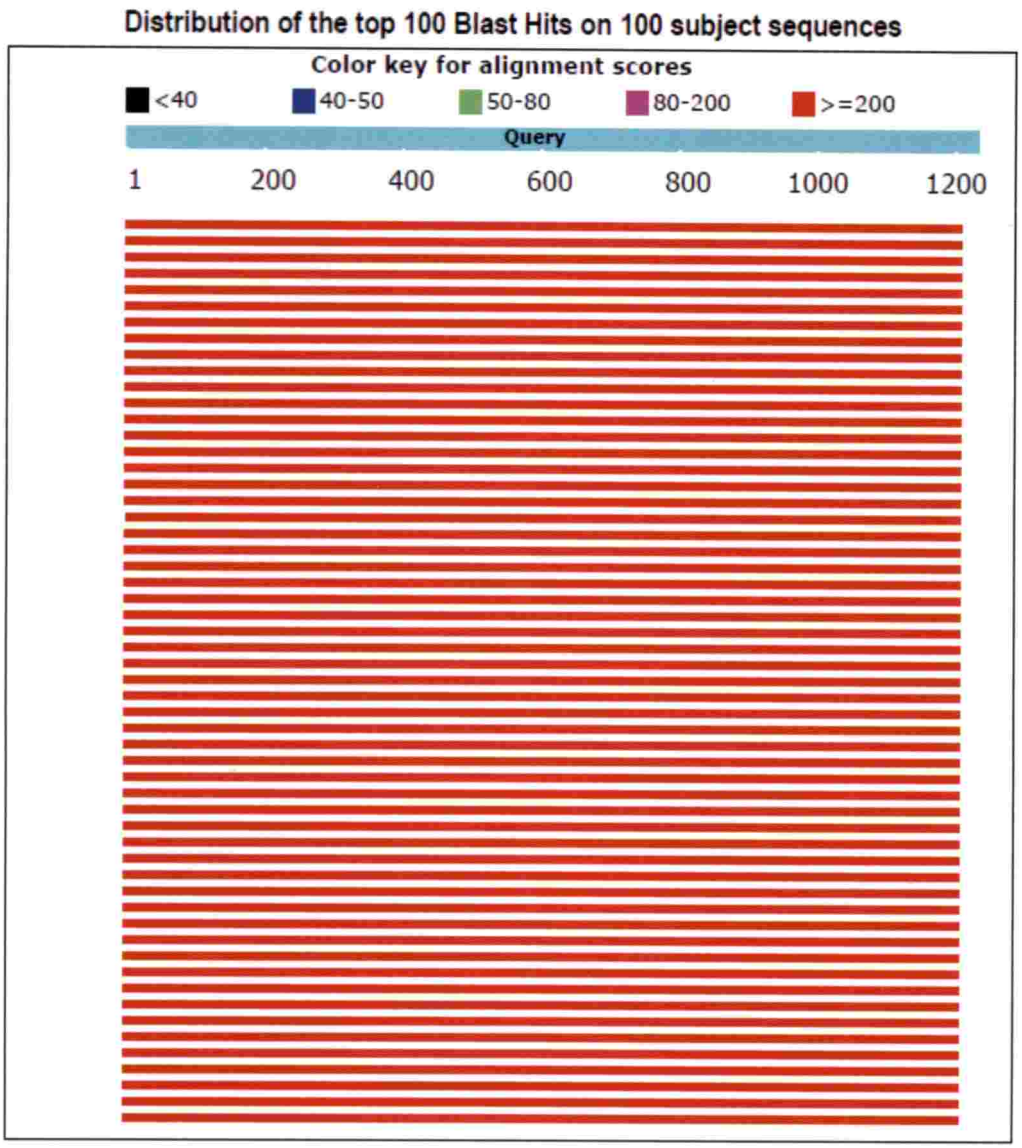
a. PPFM11



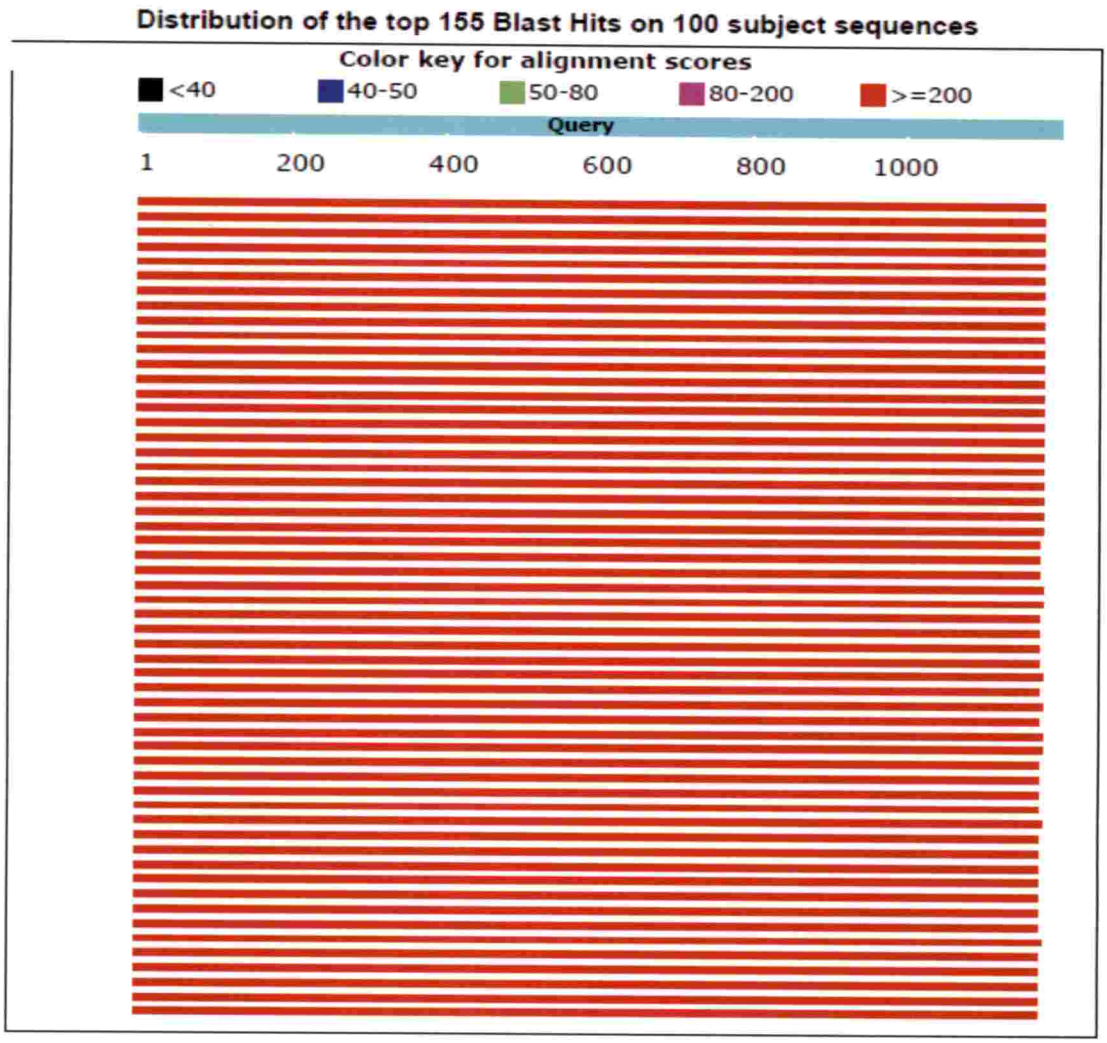
b. PPFM16


Distribution of the top 107 Blast Hits on 100 subject sequences
Color key for alignment scores
<40
40-50
50-80
80-200
>=200
Query
1
200
400
600
800
1000

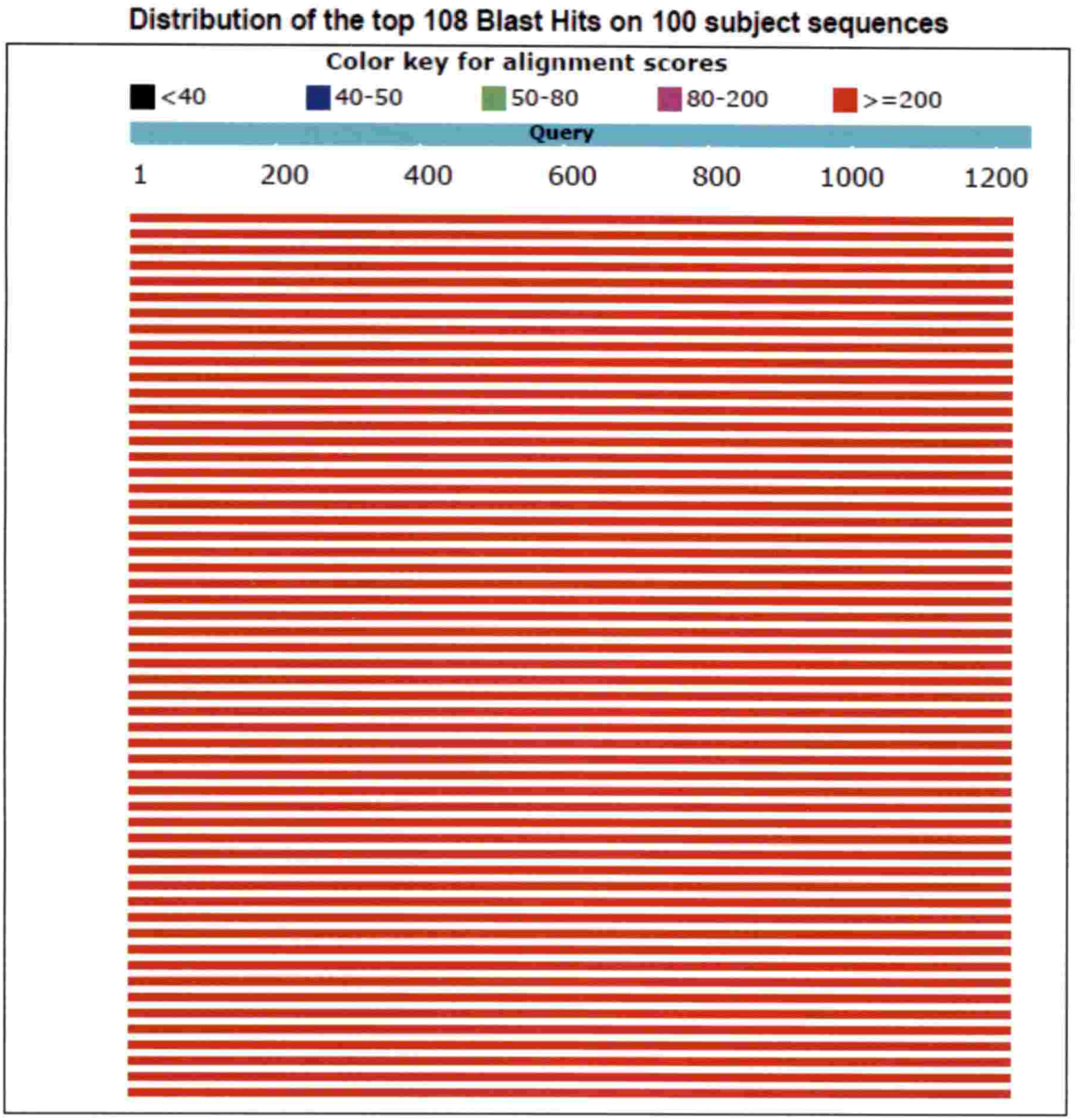
c. PPFM19


Distribution of the top 100 Blast Hits on 100 subject sequences
Color key for alignment scores
<40
40-50
50-80
80-200
>=200
Query
1
200
400
600
800
1000
1200

d. PPFM22


Distribution of the top 155 Blast Hits on 100 subject sequences
Color key for alignment scores
<40
40-50
50-80
80-200
>=200
Query
1
200
400
600
800
1000

e. PPFM35


Distribution of the top 108 Blast Hits on 100 subject sequences
Color key for alignment scores
<40
40-50
50-80
80-200
>=200
Query
1
200
400
600
800
1000
1200

ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFMs) ASSOCIATED WITH PADDY

by

NYSANTH, N. S.

(2015-11-079)

Abstract of the thesis

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requirements for the degree of

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KERALA, INDIA

2018

ABSTRACT

The study entitled "Isolation and characterization of Pink Pigmented Facultative Methylotrophs (PPFMs) associated with paddy" was conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during the period 2015-2017. The main objectives of the study were isolation, characterization and evaluation of Pink-Pigmented Facultative Methylotrophs (PPFMs) associated with paddy for antagonistic efficiency, seed germination, seedling growth and yield of paddy. .

The pink pigmented facultative methylobacteria (PPFM) were isolated from phyllosphere of paddy collected from different locations of Kerala by leaf imprint method on Ammonium Mineral Salt (AMS) agar medium supplemented with 0.5% methanol and cycloheximide. Forty six isolates were obtained from different locations and code numbers were allotted for each of the isolate. They were tentatively identified as PPFMs based on the characteristic pink pigmented colonies on AMS agar supplemented with 0.5% methanol as sole source of carbon and energy. The product developed by Tamil Nadu Agricultural University was taken as the reference culture.

Indole Acetic Acid production by the different isolates of PPFM showed wide variations ranging from 9.27 to 68.65 $\mu g\ mL^{-1}$ of culture filtrate. Maximum IAA production of 68.65$\mu g\ mL^{-1}$ of culture filtrate was recorded by PPFM35. The reference culture produced 57.39 $\mu g\ mL^{-1}$ of IAA.

All the isolates were found to produce carotenoid pigment in varied quantity ranging from 0.07 to 1.74 $\mu g\ mL^{-1}$. Among these isolates, the highest carotenoid production was recorded in PPFM16 (1.74 $\mu g\ mL^{-1}$). The reference culture recorded a total carotenoid production of 0.64 $\mu g\ mL^{-1}$.

The antagonistic efficiency of all forty seven PPFM isolates including reference culture was assessed against two important fungal pathogens of paddy namely, *Rhizoctonia solani, Pyricularia oryzae* and one bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* following dual culture method. Six out of forty

seven isolates tested inhibited *Rhizoctonia solani.* The results revealed that the isolate PPFM 10 exhibited the maximum zone of inhibition (ZOI) of 12.72 mm. The reference culture inhibited *Rhizoctonia solani* and produced a ZOI of 9.07mm. Four out of forty seven isolates tested showed antagonistic activity against *Pyricularia oryzae* with PPFM24 producing the maximum zone of inhibition of 10.00 mm. Four out of forty seven isolates tested inhibited *Xanthomonas oryzae* pv. *oryzae* and PPFM5 produced the maximum ZOI of 9.80 mm diameter.

In the study on the effect of PPFM isolates on seed germination and seedling growth both treated and uninoculated control seeds germinated on the second day of seed treatment. Maximum germination percentage of 100 was recorded in seeds treated with PPFM35. Treatment with PPFM30 recorded the maximum shoot length of 26.38 cm and maximum root length of 24.20 cm was obtained in seeds treated with PPFM22 and same isolate recorded the highest seedling vigour index of 4756.35 over the control (3037.91). The root shoot ratio of seedlings showed significant increase when seeds were treated with PPFM isolates. Maximum root shoot ratio of 0.62 was observed when seeds were treated with PPFM26 and PPFM35 compared to control (0.33).

A pot culture experiment was conducted using variety Jyothi (Ptb-39)) in completely randomized design using wetland soil. Seedlings were dipped in 2 per cent of liquid culture of the PPFM isolates before transplanting. One per cent foliar spray of the liquid culture of respective isolates was given 15 and 30 days after transplanting. Appropriate control treatments and replications were maintained. Application of PPFM isolates significantly increased growth and biomass production. Also the yield of paddy was significantly increased. PPFM11 recorded the highest grain yield of 46.30 g $hill^{-1}$ whereas the control recorded a grain yield of 33.65 g $hill^{-1}$ only. The reference culture recorded a grain yield of 38.40 g $hill^{-1}$. Physiological characterization of plants revealed significant influence of PPFM isolates on chlorophyll content, cell membrane stability and proline content of the plant compared to untreated plants.

The isolates PPFM11, PPFM16, PPF19, PPFM22 and PPFM35 were adjudged as superior isolates based on maximum germination percentage, seedling vigour index, growth promotion efficacy and grain yield of paddy. These five isolates were identified as *Methylobacterium* spp. based on morphological, biochemical and molecular characteristics.