

**ISOLATION AND CHARACTERIZATION OF PINK  
PIGMENTED FACULTATIVE METHYLOTROPHS FROM  
Coleus forskohlii AND THEIR INFLUENCE ON  
GROWTH AND TUBER YIELD**

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# CONTENTS

Sl. No.	Chapter Particulars
	CERTIFICATE
	ACKNOWLEDGEMENT
	LIST OF TABLES
	LIST OF FIGURES
	LIST OF PLATES
1.	INTRODUCTION
2.	REVIEW OF LITERATURE
	2.1 Pink pigmented facultative methylotrophs
	2.2 Phytohormone production in methylotrophs
	2.3 Methylotrophs and plant growth
3.	MATERIAL AND METHODS
	3.1 Collection of samples
	3.2 Isolation and purification of methylotrophs (PPFM)
	3.3 Characterization of methylotrophs
	3.4 Biochemical characterization
	3.5 Evaluation of the PPFM isolates for their agriculturally important properties
	3.6 Pot culture experiment
	3.7 Estimation of soil enzymes
	3.8 Population dynamics of PPFM in phyllosphere and rhizosphere of <i>Coleus forskohlii</i>
	3.9 Statistical analysis of the data
4.	EXPERIMENTAL RESULTS
	4.1 Isolation of methylotrophs from <i>Coleus forskohlii</i>
	4.2 Morphological characterization
	4.3 Biochemical characterization of methylotroph isolates
	4.4 Testing of <i>Methylobacterium</i> isolates for production of growth promoting substances and other beneficial characters
	4.5 Pot culture evaluation of the effect of selected methylotrophic isolates on growth and tuber yield of <i>Coleus forskohlii</i>
	4.6 Population dynamics of PPFM and soil enzyme activities
	4.7 Yield parameters and plant biomass production
5.	DISCUSSION
6	SUMMARY AND CONCLUSIONS
	REFERENCES

## LIST OF TABLES

Table No.	Title
1	Sources of PPFM isolates
2	Morphological characterization and Gram reaction of PPFM isolates
3	Biochemical characterization of PPFM isolates
4	Ability of PPFM isolates to utilize different carbon sources
5	Production of phytohormones, siderophores, HCN and Pi released by PPFM isolates
6	Antagonistic activity of the PPFM isolates against different plant pathogenic fungi
7	Antagonistic activity of the PPFM isolates against different plant pathogenic bacteria
8	Particulars of the selected PPFM isolates
9	Influence of the selected PPFM isolates on growth parameters of <i>Coleus forskohlii</i>
10	Effect of selected PPFM isolates on plant biomass and tuber yield of <i>Coleus forskohlii</i>
11	Phyllosphere and rhizosphere PPFM population and enzymatic activity as influenced by inoculation with selected PPFM isolates in <i>Coleus forskohlii</i>
12	Per cent increase in growth parameters by PPFM 50
13	Per cent increase in shoot biomass and tuber yield by PPFM 50

## LIST OF FIGURES

Figure No.	Title
1	Antagonistic activity of the PPFM isolates against different plant pathogenic fungi
2	Influence of the selected PPFM isolates on growth parameters of <i>Coleus forskohlii</i>
3	Phyllosphere and rhizosphere PPFM population and enzymatic activities as influenced by inoculation with selected PPFM isolates in <i>Coleus forskohlii</i>
4	Effect of selected PPFM isolates on plant biomass and tuber yield of <i>Coleus forskohlii</i>

## LIST OF PLATES

Plate No.	Title
1	Sources of samples of <i>Coleus forskohlii</i> used for isolation of PPFM isolates
2	Isolation of PPFM by leaf impringment method from different sources
3	Isolation of PPFM from stem surface of <i>Coleus forskohlii</i>
4	Isolation of PPFM from rhizoplane of <i>Coleus forskohlii</i>
5	Isolation of PPFM from root endophytes of <i>Coleus forskohlii</i>
6	Isolation of PPFM from rhizosphere soil of <i>Coleus forskohlii</i>
7	P-solubilization activity of PPFM isolates
8	Antagonistic activity of PPFM isolates
9	General view of the pot experiment
10	Effect of inoculation of PPFM isolates on growth and biomass of <i>Coleus forskohlii</i>
11	Effect of inoculation of PPFM isolates on growth and biomass of <i>Coleus forskohlii</i> (Treatment wise)
12	Effect of inoculation of PPFM isolates on root biomass of <i>Coleus forskohlii</i>
13	Effect of inoculation of PPFM isolates on root, growth and biomass of <i>Coleus forskohlii</i> (after harvest)

## LIST OF APPENDIX

<b>Appendix No.</b>	<b>Title</b>
1	Composition of media
2	Composition of stains
3	Reagents used in soil enzyme studies

# 1. INTRODUCTION

Plants are the first medicines for mankind and hundreds of plant species are harvested for their medicinal properties all over the world. In spite of modern development of sophisticated pharmaceutical chemicals to treat illnesses, medicinal plants remain an important tool for treating illness. In some regions, traditional medicines made from local plants are the only available and affordable sources for treating various ailments. World Health Organization estimates that 80 per cent of the world's population depends on traditional medicine for their health needs. In many developed countries, traditional herbal remedies are making a comeback as alternatives to modern medicine.

*Coleus forskohlii* is a perennial member of the mint (Lamiaceae) family and grows in the subtropical temperate climates of India, Nepal, Sri Lanka and Thailand. *Coleus forskohlii* is an important indigenous medicinal plant in India. It has been used in traditional ayurvedic medicine for curing various disorders. The therapeutic properties of forskohlin, the main constituent of this plant have contributed to the emergence of *C. forskohlii* as a taxon of importance in modern medicine. Forskohlin is used for the treatment of eczema, asthma, psoriasis, cardiovascular disorders and hypertension (Rupp *et al.*, 1986). In India, the crop is cultivated in different parts of Gujarat, Maharashtra, Karnataka and Tamilnadu in an area of more than 2,500 hectares for its tuberous roots.

Many of the microbes living on the phylloplane probably lead a saprophytic lifestyle, feeding on materials leached from the leaf. One such example is *Methylobacterium* sp. Pink pigmented facultative methylotrophs (PPFMs) were first identified as covert contaminants from the tissue culture of liverwort, *Scapania nemorosa* (Basile *et al.*, 1969). This bacterium provides a useful model for the unappreciated kinds of interactions between plants and bacteria that take place routinely in lab and in culture dishes (Austin and Goodfellow, 1979; Green and Bousifield 1982, 1983; Patt *et al.*, 1976). The genus *Methylobacterium* is composed of a variety of pink pigmented facultative methylotrophs *i.e.* (PPFM) and non-pigmented facultative methylotrophs *i.e.* (NPFM) which are capable of growing on C1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substrates such as C2, C3 and C4 compounds. PPFMs are ubiquitous in nature and frequently reported on various plant species. They are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves. They are also found in soils, on the seed and in the rhizosphere of a wide variety of plants, with the highest numbers being present on actively growing and meristamatic tissue, sometimes averaging  $10^4$ - $10^6$  colony forming units (cfu) per leaflet (Dunleavy, 1988).

Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins and are referred as PGPR (plant growth promoting rhizobacteria) (Holland, 1997; Ivanova *et al.*, 2001; Omer *et al.*, 2004). They have been known to improve agronomic traits like branching, seedling vigour, rooting, biomass and heat or cold tolerance in different crops (Holland, 1997).

Considering the importance of methylotrophs as well as *Coleus forskohlii*, an attempt was made to isolate, characterise, screen and select the efficient PPFM strains that can be used for inoculating *Coleus forskohlii* in the nursery with the following objectives:

1. Isolation, purification and characterization of Pink Pigmented Facultative Methylotrophs (PPFM) from phyllosphere of *Coleus forskohlii* plants.
2. To study the functional diversity of these PPFM isolates.
3. To study the effect of the efficient PPFM isolates on growth and tuber yield of *Coleus forskohlii* plants. (Pot culture studies).

## 2. REVIEW OF LITERATURE

The genus, *Methylobacterium*, is a group of strictly aerobic, facultative methylotrophic, Gram-negative and rod-shaped bacteria able to grow on one carbon compounds such as formate, formaldehyde, methanol, methylamine and a wide range of multi-carbon growth substrates as the sole source of carbon and energy (Green, 1992).

### 2.1 Pink pigmented facultative methylotrophs

The genus *Methylobacterium* belongs to alpha-2 subclass of Proteobacteria with validly published 26 species, namely *M. adhaesivum* (Gallego *et al.* 2006), *M. aminovorans* (Urakami *et al.*, 1993), *M. aquaticum* (Gallego *et al.*, 2005a), *M. chloromethanicum* (McDonald *et al.*, 2001), *M. dichloromethanicum* (Doronina *et al.*, 2000), *M. extorquens* (Bousfield and Green, 1985), *M. fujisawaense* (Green *et al.*, 1988), *M. goesingense* (Idris *et al.* 2006), *M. hispanicum* (Gallego *et al.*, 2005a), *M. isbiliense* (Gallego *et al.*, 2005b), *M. jeotgali* (Aslam *et al.*, 2007), *M. lusitanum* (Doronina *et al.*, 2000), *M. mesophilicum* (Green and Bousfield, 1983), *M. nodulans* (Jourand *et al.*, 2004), *M. organophilum* (Patt *et al.*, 1976), *M. oryzae* (Madhaiyan *et al.* 2009), *M. platani* (Kang *et al.*, 2007), *M. podarium* (Anesti *et al.*, 2004), *M. populi* (Van-Aken *et al.*, 2004), *M. radiotolerans* (Green and Bousfield, 1983), *M. rhodesianum* (Green *et al.*, 1988), *M. rhodinum* (Green and Bousfield, 1983), *M. suomiense* (Doronina *et al.*, 2000), *M. thiocyanatum* (Wood *et al.*, 1998), *M. variabile* (Gallego *et al.*, 2005b) and *M. zatmanii* (Green *et al.*, 1988).

The species of Methylotrophs are distributed in a variety of natural and man-made environments, including soil, air, dust, fresh water, marine water, water supplies, polluted soil, bathrooms, air conditioning systems, and masonry (Trotsenko *et al.*, 2001). Several species of methylotrophic bacteria are found in association with terrestrial and aquatic plants, colonizing roots, leaf surfaces, and growing buds (Lindstrom and Chistoserdova, 2002). *M. extorquens* and *M. fujisawaense* from phyllosphere of several plants (Abanda *et al.*, 2007), *M. populi*, associated with internal tissues of poplar tree (Van-Aken *et al.*, 2004), *M. nodulans* ORS2060, nodulating *Crotalaria glaucooides* (Jourand *et al.*, 2004), and *M. goesingense*, associated with *Thlapsi goesingense* (Idris *et al.*, 2006) are some of the species so far recorded as plant associated pink pigmented facultative methylotrophs (PPFMs).

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), and in response, methylotrophs may produce phytohormones such as cytokinin and auxins (Madhaiyan *et al.*, 2005). In addition, they can fix atmospheric nitrogen (Jourand *et al.*, 2004), bring about mineral phosphate solubilization (Jones *et al.*, 2007) regulate the ethylene level in rhizosphere by 1-aminocyclopropane-1-carboxylate deaminase (Madhaiyan *et al.*, 2006a), and stimulate the resistance against plant pathogens (Madhaiyan *et al.*, 2006b).

#### 2.1.1 Isolation of methylotrophs

The first *Methylobacterium* strain to be described in the literature was isolated by (Bassalik, 1913) 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 *Lolium perenne*. The first reported PPFM strain able to utilize methane was isolated by (Patt *et al.*, 1976).

Green (1992) reported that although *Methylobacterium* strains could grow from 5°C to 37°C, the temperature range of 25-30°C could be used for isolation and subsequent experiments. These organisms are fairly slow growers, often taking 2-3 days at 30°C to produce clearly visible colonies or confluent growth and often taking more than seven days for colonies to reach their maximum size of 1-3 mm in diameter on AMS medium. Growth is some times more luxuriant with a deeper pink pigmentation on glycerol-peptone (GP) agar (Green, 1992).

For isolation of PPFM strains from leaf surfaces, a leaf impression technique, using AMS or GP medium is recommended. Homogenization of whole leaves or embedding them in molten agar are alternatives, although they are not as successful as the impression technique (Green, 1992).

(Benoit *et al.*, 2004) isolated pink-pigmented, aerobic, facultatively methylotrophic bacterium, strain BJ001T from internal poplar tissues (*Populus deltoidesnigra* DN34) and identified as a member of the genus *Methylobacterium*. Phylogenetic analyses showed that the strain BJ001T was related to *M. thiocyanatum*, *M. extorquens*, *M. zatmanii* and *M. rhodesianum*. However, the strain BJ001T differed from these species in its carbon-source utilization pattern, particularly, its use of methane as the sole source of carbon and energy, this ability is shared with only one other member of the genus, *Methylobacterium organophilum*. In addition, strain BJ001T is the only member of the genus *Methylobacterium* to be described as an endophyte of poplar trees. On the basis of its physiological, genotypic and ecological properties, the isolate is proposed as novel species *M. populi* sp. nov. (type strain, BJ001T=ATCC BAA-705T=NCIMB 13946T).

A pink-pigmented, facultatively methylotrophic bacterium, designated strain JT1T, was isolated from a thallus of the liverwort *Marchantia polymorpha* L. and was analysed by using a polyphasic approach (Schauer *et al.*, 2011). Comparative 16S rRNA gene sequence analysis placed the strain in a clade with *M. adhaesivum* AR27T, *M. fujisawaense* DSM 5686T, *M. radiotolerans* JCM 2831T and *M. jeotgali* S2R03-9T, with which it showed sequence similarities of 97.8, 97.7, 97.2 and 97.4 %, respectively. However, levels of DNA–DNA relatedness between strain JT1T and these and the type strains of other closely related species were lower than 70 %. Cells of JT1T stained Gram-negative and were motile, rod-shaped and characterized by numerous fimbriae-like appendages on the outer surface of their wall (density up to 200 mm<sup>-2</sup>). Major fatty acids were C18 : 1v7c and C16 : 0. Based on the morphological, physiological and biochemical data presented, strain JT1T is considered to represent a novel species of the genus *Methylobacterium*, for which the name *M. marchantiae* sp. nov. is proposed. The type strain is JT1T (5DSM 21328T 5CCUG 56108T).

The plant phyllosphere constitutes a habitat for numerous microorganisms; among them are members of the genus *Methylobacterium* (Claudia *et al.*, 2010). Owing to the ubiquitous occurrence of methylobacteria on plant leaves, they represent a suitable target for studying plant colonization patterns. The influence of the factor site, host plant species, time and the presence of other phyllosphere bacteria on *Methylobacterium* community composition and population size were evaluated in leaf samples were collected from *Arabidopsis thaliana* or *Medicago truncatula* plants and from the surrounding plant species at several sites. The abundance of cultivable *Methylobacterium* clearly correlated with the abundance of other phyllosphere bacteria, suggesting that Methylobacteria constitute a considerable and rather stable fraction of the phyllosphere microbiota under varying environmental conditions. Automated ribosomal intergenic spacer analysis (ARISA) was applied to characterize the *Methylobacterium* community composition and showed the presence of similar communities on *A. thaliana* plants at most sites in 2 consecutive years of sampling. A substantial part of the observed variation in the community composition was explained by site and plant species, especially in the case of the plants collected at the *Arabidopsis* sites (50%). The dominating ARISA peaks that were detected on *A. thaliana* plants were found on other plant species grown at the same site, whereas some different peaks were detected on *A. thaliana* plants from other sites. This indicates that site-specific factors had a stronger impact on the *Methylobacterium* community composition than did plant-specific factors and that the *Methylobacterium*–plant association was not highly host plant species specific.

Six strains of pink-pigmented facultatively methylotrophic bacteria (PPFMs) isolated from phylloplane surfaces of different plants were analysed for the presence of triterpenoids of the hopane series (Knani *et al.*, 1994). All of them produced hopanoids in abundant quantities and contained the same compounds as the type strain of *M. organophilum*: diplopterol, 2 beta-methyldiplopterol, bacteriohopanetetrol, a tetrol glycoside and two tetrol ethers.

A pink-pigmented, aerobic, facultatively methylotrophic bacterial strain, CBMB27<sup>T</sup>, isolated from leaf tissues of rice (*Oryza sativa* L. 'Dong-Jin'), was analysed using a polyphasic taxonomic approach (Madhaiyan *et al.*, 2009).

Comparative 16S rRNA gene sequence-based phylogenetic analysis placed the strain in a clade with the species *Methylobacterium oryzae*, *Methylobacterium fujisawaense* and *Methylobacterium mesophilicum*; strain CBMB27<sup>T</sup> showed sequence similarities of 98.3, 98.5 and 97.3%, respectively, to the type strains of these three species. Based on the results of 16S rRNA gene sequence analysis, DNA–DNA hybridization, and physiological and biochemical characterization, which enabled the isolate to be differentiated from all recognized species of the genus *Methylobacterium*, it was concluded that strain CBMB27<sup>T</sup> represented a novel species in the genus *Methylobacterium* for which the name *Methylobacterium phyllosphaerae* sp. nov. was proposed (type strain CBMB27<sup>T</sup> =LMG 24361<sup>T</sup> =KACC 11716<sup>T</sup> =DSM 19779<sup>T</sup>).

Methanol-ammonium salts agar surfaces impressed with leaf disks or whole leaves of green plants, resulted in growth of methylophilic colonies in a pattern reflecting the distribution of the organisms on the leaf (William, 1985). The predominant methylophilic encountered and isolated were pink-pigmented, facultatively methylophilic (PPFM) bacteria. The relative abundance of PPFMs on both dorsal and ventral leaf surfaces of over 50 field and glasshouse collected plant species, ranged from 0.1 colony forming units/cm<sup>2</sup> to 500 colony forming units/cm<sup>2</sup>. The method is also useful for study of methylophilic distribution on other surfaces. Painted or unpainted metal, wood, glass, plastic, brick, stone and concrete showed on the average, 0.14–1.9 cfu/cm<sup>2</sup> of PPFMs.

Current interest in the chemistry of the atmosphere has led to the identification of methanol as one of the major organic compounds in forest air and in the troposphere (Ray *et al.*, 2005). Analysis of the emissions of volatile organic compounds from leaves has revealed that most plants emit methanol, especially during early stages of leaf expansion; it is probably produced as a by-product of pectin metabolism during cell wall synthesis, and a fraction of this pool is then emitted through stomata during transpiration. There is also evidence that leaf methanol supports an abundant population of epiphytic bacterial methylophilic. Application of methanol to leaves enhances the growth and yield of C<sub>3</sub> plants, although the mechanism responsible for this phenomenon is uncertain.

### 2.1.2 Characterization of *Methylobacterium* strains

Except for some, most of the *Methylobacterium* strains grow slowly on nutrient agar. After seven days of incubation at 30°C, colonies on MMS agar are a more uniform pale pink. The pigment is non-diffusible, non-fluorescent and probably is a carotenoid (Downs and Harrison, 1974). The optimum growth temperature for all *Methylobacterium* strains is in the range of 25 to 30°C. Some strains will grow at 51°C or less and some will grow at or above 37°C. Although, growth is optimal around neutrality, some strains can grow at pH 4.0 and some at pH 10.0. Growth factors are not required by any strain (Green, 1992).

#### 2.1.2.1 Morphological characteristics

All *Methylobacterium* strains are rods (0.8–1.0 × 1.0–8.0 μm), which occur singly or occasionally in rosettes (Patt *et al.*, 1974). They are often branched or pleomorphic, especially in older stationary phase cultures. They exhibit polar growth or a budding morphology. All strains are motile by a single polar, subpolar or lateral flagellum, although some strains are not vigorously motile (Heumann, 1962). They are Gram negative, although many strains stain as Gram variable (Green and Bousfield, 1982).

#### 2.1.2.2 Biochemical characteristics

All *Methylobacterium* strains are catalase and oxidase positive. They are chemoorganotrophs and facultative methylophilic capable of growing on a variety of C<sub>1</sub> compounds. Urease is produced by all strains and some strains have weak lipolytic activity.

#### 2.1.2.3 Carbon utilization tests

Species within the genus *Methylobacterium* are differentiated mainly by the pattern of compounds they utilize as carbon and energy source. Green and Bousfield (1983) reported that the compounds that were used by most (≥ 95 per cent) strains of *Methylobacterium* include, methylamine, trimethylamine, acetate, citrate, L-glutamate, D-glucose, D-xylose, fructose and betaine.

Summary of the results of tests for carbon source utilization 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 (Green and Bousifield, 1983).

Bacteria belonging to the genus *Methylobacterium* are characterized by being able to rely on methanol as a sole carbon and energy source and by presenting a more or less intense pink reddish pigmentation (Zahra *et al.*, 2004). These bacteria, are common inhabitants of the phyllosphere and are found in many other environmental samples. Since, they grow slowly, they are often overlooked and their impact on phyllosphere microbial communities and on the plants harboring them is not well studied nor has their ecology been elucidated. In a survey of PPFM colonization in three different agricultural sites, PPFM populations were identified on both red clover and winter wheat, but red clover was more consistently colonized. Isolations from collected leaves showed PPFM populations to decrease from spring towards summer, but they increased again towards the end of the cropping season. Isolates from red clover readily colonized winter wheat leaves and *vice versa* in greenhouse experiments, but population sizes were dependent on the application procedure. Tested isolates had also good potential to colonize the rhizosphere, especially after seed inoculations. Confocal scanning laser microscopy showed gfp-tagged isolates to colonize the surface of clover leaves by forming large aggregates.

## 2.2 Phytohormone production in methylotrophs

Growth regulators play a crucial role in enhancing the vigour and increasing the productivity of crop plants. Recently, as an alternative to the plant growth regulators, researchers are now focusing on microbial sources of growth regulators in their context of sustainability and cost of chemicals (Thangamani and Sundaram, 2005). The epiphytic and soil microorganisms like *Azospirillum*, *Rhizobium* and *Pseudomonas* are able to synthesize and secrete plant growth promoting substances due to which they may exert beneficial effects on plants and influence the growth of plants. One such organism is the facultative *Methylobacterium* sp.

Soumya *et al.* (2011) Screened fourteen pink pigmented facultative methylotroph (PPFM) inhabiting phyllosphere of different tropical plants for plant growth hormone production and their effect on seed germination and seedling growth of cowpea (*Vigna unguiculata* (L.) Walp). The best isolates were selected for studying their effect on *in vitro* culture of cowpea. The isolates were found to induce direct morphogenesis of cowpea explants in hormone-free Murashige and Skoog medium.

The response of cowpea to *in vitro* culture varied with the type of *explant* used. The regeneration percentage was comparatively more in epicotyl than in hypocotyl *explants*. The population of PPFMs in regenerated plantlets ranged from 10.5 to 18.2 cfu g<sup>-1</sup> on fresh weight basis indicating a stable association between the bacteria and the plantlets. There was significant increase in soluble protein and chlorophyll content of the regenerated plantlets over controls.

Shanmugam *et al.* (2011) isolated pink pigmented facultative methylotrophs (PPFM) from water samples of Cooum and Adyar rivers in Chennai and soil samples of forests located in various districts of Tamil Nadu, India and were screened for cellulase production using carboxymethylcellulose agar (CMC agar) medium. The strains showed wide variations in the production of clearing zones around the colonies on CMC agar medium flooded with Congo red. CMC case and filter paper assays were used to quantitatively measure the cellulase activity of 13 PPFM strains.

Among the strains, *M. gregans*, MNW 60, MHW 109, MSF 34, and MSF 40 showed cellulolytic activity ranging from 0.73 to 1.16 U mL<sup>-1</sup> with wide temperature (35-65°C) and pH (5 to 8) tolerance. SDS-PAGE analysis of the crude enzyme of PPFM strain MNW 60 exhibited several protein bands, and zymogram analysis revealed two dimeric cellulase bands with molecular mass of ~92 and 42 kDa. Scanning electron microscopic studies revealed significant morphological differences between the cells grown in normal and CMC amended medium.

The strain MNW 60 was identified as *Methylobacterium sp.* based on biochemical, physiological, and morphological analyses, and the methylotrophic nature was authenticated by the presence of *mxoF* gene, encoding methanol dehydrogenase as a key indicator enzyme of methylotrophs with 99% similarity to *Methylobacterium lusitanum*. With the 16S ribosomal RNA sequence showing 97% similarity to *M. lusitanum* strain MP2, this can be proposed as a novel taxon of the genus *Methylobacterium*. The study forms the first detailed report on the extracellular cellulase production by pink pigmented *Methylobacterium sp.* and it is expected that this might be the basis for further studies on cellulase production by PPFMs to explore the molecular mechanism, strain improvement, and large-scale cellulase production for its application.

### 2.2.1 IAA

It is well established that many soil and plant associated bacterial groups are able to synthesize the phytohormone indole-3-acetic acid (IAA) due to which they influence the growth of plants (Felt *et al.*, 1987). The list of species synthesizing IAA is steadily growing and presently includes both gram negative and gram positive bacteria. Many of these also can produce and excrete in their cultures more than one hormone type. Different strains of *Methylobacterium* were found to produce and secrete the phytohormone auxins, in amounts of 3-100  $\mu\text{g ml}^{-1}$  (Ivanova *et al.*, 2001). However, addition of the amino acid tryptophan, a precursor of auxin, was required for the stimulation and continuous biosynthesis of bacterial indole-3-acetic acid (Ivanova *et al.*, 2001). In a detailed study, it was shown that the rate of auxin production is strain-dependent and therefore a "species-specific" characteristic. Based on two independent methods (a colorimetric technique and thin layer chromatography), (Hornschuh *et al.*, 2006) have shown that the "domesticated" type strain *M. mesophilicum* that were maintained in the laboratory since 1973, produced only about 1/3 the amount of auxin compared with two unidentified, plant-associated "wild-type" isolates of the genus *Methylobacterium*.

IAA is presumably synthesized by methylobacteria through indole-3-pyruvic acid (Ivanova *et al.*, 2001). (Omer *et al.*, 2004) reported the presence of indole-3-acetic acid (IAA) in supernatants of pink-pigmented facultative methylotrophic (PPFMs) bacterial cultures. Three out of the 16 isolates tested showed a positive reaction in a colorimetric assay. The presence was further unambiguously confirmed by high-performance liquid chromatography in combination with NMR. (Suresh Reddy, 2002) observed an increased plant growth and biomass and yield of groundnut due to the production of both auxins and cytokinins. Further, he also observed balanced growth between root and shoot growth of groundnut due to the action of both auxin and cytokinin respectively. Long (2000) suggested that the methylotrophic bacteria promote the growth of the seedling by synthesizing not only cytokinins but also other phytohormones like auxins.

### 2.2.2 GA

Gibberellins are a group of plant growth regulators which acts by modifying the plant morphology by extending the plant tissue particularly the stem which induces the uptake of minerals (Atzorn *et al.*, 1988). GA induces the uptake of minerals like K and Ca. It also increases the chlorophyll content, soluble sugars and protein content of the plants. Besides, there enhanced the growth and faster elongation rate in shoot were exhibited due to induction of active hairy root zone (Hamida and Elkomy, 1998). Anurajan (2003) reported GA production by *Methylobacterium sp* and the amount of GA production was found to vary with strains ranging from 10. 9  $\mu\text{g}$  to 106. 97  $\mu\text{g ml}^{-1}$  of the culture broth.

### 2.2.3 Cytokinin

Cytokinins are a class of compounds that are defined by their ability to stimulate cell division in plants. They are found throughout the plant, but are most abundant in actively growing tissues. Their isolation from corn, following the discovery of their effect of plant cells in culture, led to the assumption that cytokinins are endogenously produced plant growth regulators. This assumption gained the status of fact, although it was never proven. Conversely, cytokinin production by plant associated microorganisms is well documented. Root tissue is currently favoured as the major site of cytokinin biosynthesis.

Roots contain high concentration of cytokinins, but it is not clear whether they are endogenous products. Many root associated bacteria are demonstrated cytokinin producers, including *Bradyrhizobium*, *Arthrobacter*, *Streptomyces*, *Frankia* sps. and mycorrhizal fungi. The ubiquity of microbes associated with roots and their contribution to root function is better understood now than at any time since the discovery of cytokinins.

When PPFM population in seeds are reduced, seed germination declines. This effect could be reversed by inoculating seeds with PPFMs or by applying cytokinins (Holland and Polacco, 1994). Recently, it has been demonstrated that free-living PPFMs produced zeatin and zeatin riboside. Seedlings growing with reduced number of PPFMs have stunted roots. Inoculation of such plants with PPFMs or application of cytokinins to such plants restored normal roots development (Holland, 1997).

Presence of PPFMs in adequate quantities in the growing tissues leads to the cytokinin production. Cytokinins are said to act as signal molecules and initiate the plant cell to divide and this leads to the demethylation of pectin and the release of methanol. This speculative theory doubts the origin of cytokinins, which were formerly thought to be of plant origin. This theory gains credit due to the fact that cytokinins are not systemic in nature and are only having localized effects (Holland, 1997).

#### 2.2.4 Siderophore production

Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron. They sequester ferric iron, whose concentration is very low in well aerated soils, in a form that cannot be utilized by the pathogen, thereby reducing its number and/or activity. The ability to sequester iron provides a competitive advantage to microorganisms. Siderophores chelate ferric ions with a high specific activity and serve as vehicles for the transport of ferric iron into microbial cell (Neilands, 1986).

Holland (1997) has worked on the role of PPFM in iron nutrition of *Vicia faba*. *Methylobacterium mesophilicum* was found to produce siderophores with methanol or galactose as the carbon source under iron-limited conditions.

#### 2.2.5 P-Solubilization

Shanmugam *et al.* (2011) screened thirteen pink-pigmented facultative methylotrophic (PPFM) strains isolated from Adyar and Cooum rivers in Chennai and forest soil samples in Tamil Nadu, India, along with *Methylobacterium extorquens*, *M. organophilum*, *M. gregans*, and *M. komagatae* for phosphate solubilization in plates. P-solubilization index of the PPFMs grown on NBRIP—BPB plates for seven days ranged from 1.1 to 2.7. The growth of PPFMs in tricalcium phosphate amended media was found directly proportional to the glucose concentration. Higher phosphate solubilization was observed in four strains MSF 32 (415 mg l<sup>-1</sup>), MDW 80 (301 mg l<sup>-1</sup>), *M. komagatae* (279 mg l<sup>-1</sup>), and MSF 34 (202 mg l<sup>-1</sup>), after seven days of incubation. A drop in the media pH from 6.6 to 3.4 was associated with an increase in titratable acidity. Acid phosphatase activity was more pronounced in the culture filtrate than alkaline phosphatase activity. Adherence of phosphate to densely grown bacterial surface was observed under scanning electron microscope after seven-day-old cultures. Biochemical characterization and screening for methanol dehydrogenase gene (*mxoF*) confirmed the strains as methylotrophs. The *mxoF* gene sequence from MSF 32 clustered towards *M. lusitanum* sp. with 99 per cent similarity. This study forms the first detailed report on phosphate solubilization by the PPFMs.

#### 2.2.6 Methylophs and soil enzyme activity

The major parameters of soils fertility are their biological properties. Among these, special emphases is given to the enzyme activity. Many researches established that the activity of soil enzymes can be an additional diagnostic index of soil fertility and it changes as a result of human activity (Evgenia and Dadenko, 2006).

Urease, phosphatase and dehydrogenase are recognized as important soil enzymes. Measurement of dehydrogenase activity provides an index of catabolic activity of the soil. Urease is involved in the transformation of urea. As microorganisms are important sources of soil enzymes, the measurement of activities of these enzymes have often been used as an index of microbial activity (Dadenko, 2005).

The PPFM are known to produce urease enzyme (Dunleavy, 1988). Madhaiyan *et al.* (2009) reported that the co-inoculation of the bacterial strains increased the activity of nitrogenase, urease and phosphatase enzymes in soil when compared to uninoculated control or individual inoculations.

Meenakshi and Savalgi (2009) observed an increased soil enzymatic activities *viz.*, urease, dehydrogenase and phosphatase in the soybean rhizosphere that received both seed inoculation and foliar spray of *Methylobacterium*.

### 2.3 Methyloprophs and plant growth

Holland and Polacco (1992) reported a method for treating plants for increasing productivity of a plant by spraying PPFM bacteria on a plant. The invention also relates to increasing productivity of a plant under stress by applying PPFM to a plant and subsequently applying an aqueous solution containing methanol to the plant. The effect of PPFMs on methanol induced yield increases was examined (Munsanje *et al.*, 1996). In field trails, treatments of methanol and/or urea were applied to test plots of soybean. PPFM populations were determined on treated and control plants 10 days after spraying. Yields were determined after harvesting the plants. Increase in the number of PPFMs correlated with the yield increases obtained under each of the treatment regimes. Doubling of the PPFM population in response to the methanol/urea spray treatments translated into a 45 per cent increase in yield over control plants with normal PPFM populations. Yield was determined by dry weight measurements of the plant two weeks after spraying. The results showed that only in the presence of PPFMs the application of methanol resulted in increased growth (Munsanje, 1996). PPFMs could be used as seed inoculum or in seed coatings designed to enhance germinability, storability or vigour of the seeds.

Another interesting effect of PPFMs is on seed germination. The pink pigmented facultatively methyloprophic (PPFM) bacterium influences seed germination and seedling growth by producing the plant growth regulator zeatin and related cyotkinins. Germination of both fresh and aged seed is enhanced by treatment with PPFMs. A number of options for applying treatments of the bacteria to seeds were tried and these include use of freeze-dried inoculates. Vacuum infiltration of seed and introduction of the bacteria during seed imbibitions.

All these methods are effective in the process of applying PPFM bacteria to seeds and later arraying the bacterial populations. Other experiments involving aged seeds showed us that assaying PPFM populations in seed can be used to predict seed storability (Holland and Polacco, 1994). Similar studies of the seed germination and development of plants treated with PPFM's and PPFM spent medium where the inoculated plants performed better than the uninoculated. Reduction in the population of the PPFM's in the seed coat also led to reduced germination levels speculating the role of PPFM's on seed germination. The population of PPFM's range from 10<sup>5</sup> to 10<sup>9</sup> cfu per gram of fresh tissue and they could not be dismissed as accidental visitors to the plant surface. The fact that their numbers are more in the actively growing regions and their numbers are regulated by dilution as the plant tissue on which they are living expands away from the growing tissue are also worth considering, pink pigmented, facultatively methyloprophic bacteria (PPFM's) are normally associated with seeds and leaves of soybean and all other plants (Freyermuth *et al.*, 1996).

Madhaiyan *et al.* (2005) studied the effect of pink-pigmented facultative methyloprophic bacteria on germination, growth and yield of sugarcane clone Co86032. They observed that true seeds inoculated with PPFMs had a higher germination per cent and rate of germination than the control. A combined treatment of seed imbibitions, soil application and phyllosphere spray increased specific leaf area, plant height, number of internodes, and cane yield. Immunological determination of cytokinin in young and mature leaves significantly increased when the epiphytic population on the leaf surface was increased. Trends in sugar qualities in the form of pol (sucrose %) in cane, brix % in cane and commercial cane sugar were similar to that of cane yield. These effects might be mediated by the production or synthesis of plant hormones.

Co-inoculation of *Methylobacterium oryzae* with *Azospirillum. brasilense* CW903 or *Burkholderia pyrrocinia* improved the N and P concentration of plants, while the results varied among the plant species tested (Madhaiyan *et al.*, 2009). Radha *et al.* (2009) observed increased root and shoot N in soybean plants that received *Methylobacterium* inoculations.

Investigations were carried out to study the diversity of native AM fungi and Pink Pigmented Facultative Methyloph (PPFM) from four ecological zones of Northern Karnataka and their growth promoting potentials in grapevine (Jones, 2010). The authenticity of PPFM isolates was confirmed by screening for the presence of *mxoF* gene by amplifying a single fragment of 555 bp in all the native isolates. To understand the genetic diversity in these isolates, restriction digestion of amplified 16S rDNA gene was done. The sequence analysis of the 16S rDNA has clearly indicated PPFM 365, 120, 61 and 242 to be very close to *M. radiotolerance* JCM 2831 (D32227). Further, PPFM G4 isolated from the rhizosphere of vineyards showed close homology with *M. fujisawwaense* (AJ 250801). PPFM 23 isolate from the saline soils of Northeastern dry zone had very close homology with *M. thiocyanatum* (U58018). However, PPFM 117 with MPS activity isolated from the Western Ghats emerged in a separate clad. The study has brought out the diversity of AMF and PPFM and their importance in the growth promotional ability in the grapevine.

### 3. MATERIAL AND METHODS

The present investigation was carried out during the year 2011-12 in the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, on studies of Methylootrophs and their beneficial effects on *Coleus forskohlii*. The details of the materials used and methods followed in the present study are detailed in this chapter.

#### 3.1 Collection of samples

Samples of leaves, roots, tubers, and soils were collected from seedlings of *Coleus forskohlii* grown in different villages such as Nandihalli, Naganahatti, Rajahamsagada, Tarihalli, Hidakal, of Belgaum district, Dharwad and Bangalore (GKVK). The samples were brought to the laboratory in sterile polythene bags and stored at 4°C to isolate methylootrophs later. The sampling locations are depicted in Table 1 and Plate 1.

#### 3.2 Isolation and purification of methylootrophs (PPFM)

Methanol mineral salts (MMS) or Ammonium Mineral Salts (AMS) medium (Whittenbury *et al.*, 1970) is a selective medium for isolation of methylootrophs. The MMS medium was sterilized by autoclaving at 121°C for 20 minutes and cooled to 50°C. Filter sterilized vitamin solution (Colby and Zatman, 1973) was added, along with 0.1-0.2 per cent v/v sterile methanol. The pH of the medium was adjusted to pH 7.0. Solidified media (MMS agar) was prepared by the addition of 1.8 per cent purified agar before autoclaving.

##### 3.2.1 Isolation techniques

Leaf imprinting technique (Corpe *et al.*, 1985)

Imprintings of the upper and lower surface fresh leaf samples, tuber surface, stem surface and root surface were made on the solidified medium separately and incubated at 28±2°C for five days.

Serial dilution technique: One gram sample of soil, root, tuber was ground using a pestle and mortar, serially diluted upto 10<sup>-6</sup> dilutions and one ml each of the aliquots from 10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup> dilutions was transferred to sterile petridishes. The AMS medium was sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. Filter sterilized cyclohexamide (30 µg ml<sup>-1</sup>) antibiotic solution and 0.5 per cent (v/v) methanol were incorporated into the sterilized AMS medium before plating. After plating with AMS medium, the plates were incubated in an inverted position for 3-5 days at 30°C. Characteristic pink colonies growing over the medium were identified (Corpe and Rheem, 1989). Further, the methylootrophs were purified by the streak plate method and well isolated colonies on the plates were preserved on AMS agar slants. Isolates were maintained on AMS slants at 4°C in a refrigerator for further use. The details of the location from where they were isolated are given in Table 1.

#### 3.3 Characterization of methylootrophs

All the isolates were examined for their cell size, cell shape, motility and Gram reaction.

##### 3.3.1 Cell size

The 72 h grown facultative methylootrophic isolates were observed microscopically and their sizes measured.

##### 3.3.2 Cell shape (Becking, 1974)

The 72 h grown cells were stained with methylene blue and their shape determined under a microscope.

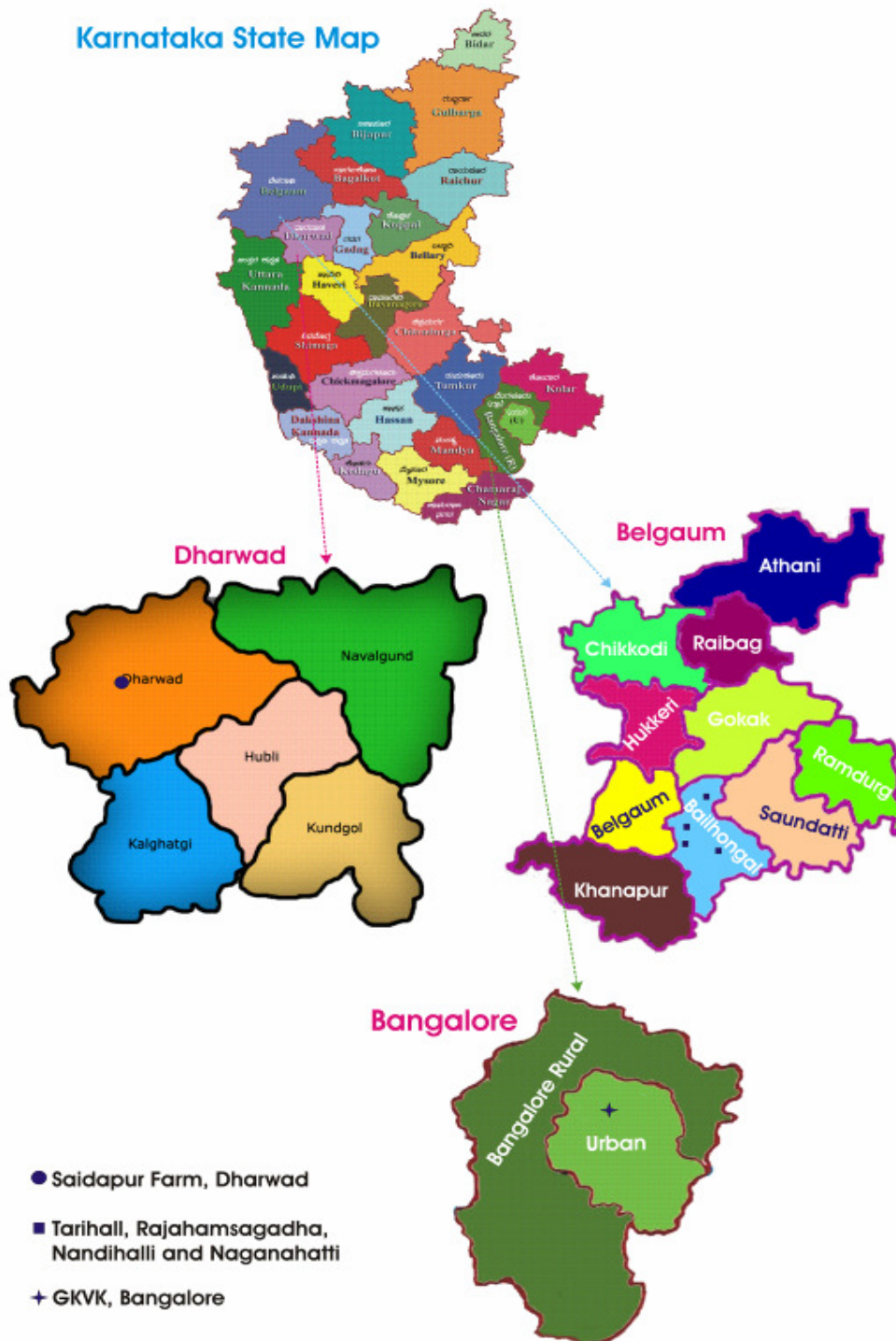
##### 3.3.3 Motility studies

The 72 h grown PPFM isolates were observed microscopically using a cavity slide for their motility and recorded.

**Table 1: Sources of PPFM isolates**

Sl.no.	Code no. of the isolate	Place of isolation	Habitat
1	PPFM 1	Naganahatti 3	Stem
2	PPFM 2	Naganahatti 3	Stem
3	PPFM 5	Nandihalli 3	Stem
4	PPFM 8	Naganahatti 2	Rhizoplane
5	PPFM 9	Nandihalli 1	Rhizoplane
6	PPFM 10	Nandihalli 1	Rhizoplane
7	PPFM 14	Nandihalli 2	Phyllospher dorsal
8	PPFM 15	Naganahatti 4	Rhizoplane
9	PPFM 16	Naganahatti 4	Rhizoplane
10	PPFM 17	Naganahatti 4	Rhizoplane
11	PPFM 20	Nandihalli 1	Rhizoplane
12	PPFM 22	Nandihalli 3	Tuber surface
13	PPFM 24	Nandihalli 4	Tuber surface
14	PPFM 26	Nandihalli 2	Stem
15	PPFM 27	Nandihalli 2	Stem
16	PPFM 28	Nandihalli 1	Stem
17	PPFM 30	Rajahamsagada 2	Endophyte
18	PPFM 32	Tarihalli 2	Phyllosphere ventral
19	PPFM 36	Naganahatti	Root Endophyte
20	PPFM 37	Nandihalli 2	Root Endophyte
21	PPFM 38	Rajahamsagada 2	Root Endophyte
22	PPFM 39	Naganahatti 2	Root Endophyte
23	PPFM 40	Naganahatti 2	Root Endophyte
24	PPFM 41	Nandihalli 4	Root Endophyte
25	PPFM 42	Nandihalli 4	Root Endophyte
26	PPFM 45	Rajahamsagada 2	Root Endophyte
27	PPFM 49	Nandihalli 1	Root Endophyte
28	PPFM 50	Nandihalli 1	Root Endophyte
29	PPFM 54	Naganahatti 1	Root Endophyte
30	PPFM 58	Nandihalli 2	Root Endophyte
31	PPFM 59	Nandihalli 2	Root Endophyte
32	PPFM 60	Nandihalli 2	Root Endophyte
33	PPFM 81	Dharwad 2	Leaf ventral
34	PPFM 83	Dharwad 2	Endophyte
35	PPFM 100	Nandihalli 1	Phyllosphere dorsal
36	PPFM 117	Hidakal (organic plot)	Tuber
37	PPFM 119	Hidakal (inorganic plot)	Tuber
38	PPFM 120	Hidakal (inorganic plot)	Tuber
39	PPFM 132	Hidakal (organic plot)	Stem
40	PPFM 138	Hidakal (inorganic plot)	Tuber
41	PPFM 148	Dharwad	Root Endophyte
42	PPFM 151	Hidakal (general 2 plot)	Root Endophyte
43	PPFM 156	Hidakal(general 2 plot)	Phyllosphere dorsal
44	PPFM 162	Hidakal (inorganic plot)	Phyllosphere dorsal
45	PPFM 165	Hidakal (general 1 plot)	Phyllosphere dorsal
46	PPFM 168	Hidakal (organic plot)	Phyllosphere dorsal
47	PPFM 170	Tarihalli 3	Stem
48	PPFM 184	Bangalore (GKVK farm)	Phyllosphere ventral
49	PPFM 188	Bangalore (GKVK farm)	Stem
50	PPFM 189	Bangalore (GKVK farm)	Soil

## Karnataka State Map



**Plate 1: Sources of samples of *Coleus forskohlii* used for isolation of PPFM isolates**

Plate 1. Sources of samples of *Coleus forskohlii* used for isolation of Prfm Isolates

### 3.3.4 Gram reaction (Rangaswami and Bagyaraj, 1993)

Gram staining was carried out for all the methylotroph isolates and the observations recorded.

## 3.4 Biochemical characterization

### 3.4.1 Catalase activity (Gerhardt *et al.*, 1981)

A loopful of 24 h old culture of PPFM isolates maintained on AMS agar slant was transferred to a glass test tube containing 0.5 ml distilled water and mixed thoroughly with 0.5 ml of 3 per cent hydrogen peroxide solution, observed for effervescence and recorded the results.

### 3.4.2 Citrate utilization test (Seeley and Vandemark, 1981)

The PPFM isolates were inoculated into test tubes containing Simmons citrate agar medium and incubated for 48 h at 35°C. Simmons citrate agar contains citrate as its only carbon and energy source. The presence of growth and change of colour from green to blue due to pH change indicated positive reaction.

### 3.4.3 Oxidase test (Cappuccino and Sherman, 1996)

The PPFM isolates were streaked on trypticase soy agar medium and incubated at 37°C in an inverted position for 48 h. After the incubation period, 2-3 drops of P-amino dimethyl aniline oxalate solution was added on the streaked area and the plates were observed for the color change from pink to maroon and finally to purple within 30 seconds indicating positive reaction.

### 3.4.4 Urease test (Cappuccino and Sherman, 1996)

Urease test was performed in 5 ml urea broth in test tubes containing phenol red (pH 6.8) as the pH indicator. The cultures were inoculated into the sterilized urea broth and incubated for 24 h. The development of red color indicates a positive reaction for the test.

### 3.4.5 Carbon source utilization test (Green and Bousifield, 1983)

AMS liquid medium was prepared with the following carbon compounds *viz.*, glucose, D-galactose, dichloromethane, chloroform and glycerol substituted for methanol at 0.5 percent level and inoculated with one per cent (v/v) standard inoculum ( $10^9$  cfu ml<sup>-1</sup>) and incubated in an incubator shaker (150 rpm) at 30°C for 5 days and compared with a negative control containing no added carbon source.

### 3.4.6 Amount of TCP solubilized by the strain

The PPFM isolates growing on Pikovskaya's agar were further examined for their ability to release Pi from TCP in the broth medium. One ml of overnight culture of each isolates was inoculated to 50 ml of Pikovskaya's broth in three replicates. All the inoculated flasks were incubated for ten days at 28 -30°C on a temperature controlled shaker. The Pi released in the broth was estimated at 10 days of incubation from flasks at each stage in comparison with the set of uninoculated controls. The broth cultures were centrifuged at 10,000 rpm for ten minutes in Sigma centrifuge to separate the supernatant from the cell growth and insoluble phosphate. The available P content in the supernatant was estimated by phosphomolybdc blue color method (Jackson, 1973).

### 3.4.6 Antimicrobial activity (*in vitro*)

The PPFM isolates were screened for their ability to inhibit the growth of *Sclerotium rolfsii* following the dual culture assay (Ganesan and Gnanamanikyam, 1987). *Methylobacterial* isolates were tested for biocontrol potential against fungal pathogen *Fusarium oxysporum* (Poorniammal *et al.*, 2009). Zone of inhibition (ZOI in dia in mm) of the pathogen by individual isolates was measured and recorded.

## 3.5 Evaluation of the PPFM isolates for their agriculturally important properties

### 3.5.1 Indole acetic acid production

All the isolates were examined for IAA production qualitatively. The IAA production by PPFM isolates which were positive in the initiative assay was determined following the method of Ivanova *et al.* (2001). One hundred ml quantities of AMS liquid medium was dispensed into 250 ml erlenmeyer flasks and sterilized at 15 psi for 15 min. Freshly prepared, filter sterilized solution of L-tryptophan was added to a final concentration of  $100 \text{ mg l}^{-1}$ , one ml of the standard inoculum ( $10^9$  cells  $\text{ml}^{-1}$ ) of PPFM isolates was inoculated to each flask and incubated at room temperature in a shaker. In order to avoid photo inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 7 days of incubation period, 25 ml of the sample was withdrawn and the cells were spun at 5000 g for 15 min in a centrifuge for quantitative estimation of IAA.

#### 3.5.1.1 Quantitative estimation of IAA production in PPFM isolates by spectrophotometric method (Sy *et al.*, 2001)

A known quantity of the sample was taken (0.5ml) in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Saplér's reagent and incubated in darkness for 1 hr at  $28^\circ\text{C}$ . The intensity of the pink color developed was read in a spectrophotometer at 540 nm. By referring to a standard graph prepared with chemical grade indole-3-acetic acid, the quantity of IAA in the sample was determined and expressed as  $\mu\text{g ml}^{-1}$  of culture filtrate.

### 3.5.2 Gibberellic acid (GA) production

#### 3.5.2.1 Extraction of gibberellins (Tien *et al.*, 1979)

Three to four days old PPFM culture was centrifuged for 15 min at 10,000 rpm and the supernatant taken. The cell pellet was re-extracted with phosphate buffer (pH 8.0) and again centrifuged. Both the supernatants were pooled, acidified at pH 2.5 using 5 N hydrochloric acid and partitioned with equal volumes of ethyl acetate for five times. The ethyl acetate phase was dried at  $32^\circ\text{C}$  and the residue redissolved in 2 ml distilled water containing 0.05 per cent Tween 80.

#### 3.5.2.2 Estimation of gibberellins (GA) (Mahadevan and Sridhar, 1982)

Fifteen ml of ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added. After 2 min, 2 ml of potassium ferrocyanide solution was added and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 per cent hydrochloric acid and the mixture was incubated at  $20^\circ\text{C}$  for 75 minutes. The blank was prepared with 50 per cent hydrochloric acid. The absorbance was measured at 254 nm in a spectrophotometer. From the standard graph using standard gibberellic acid solution the amount of GA produced by the FM isolates was calculated and expressed as  $\mu\text{g ml}^{-1}$  broth.

### 3.5.3 Siderophore production (Schwyn and Neilands, 1987)

For the assay of siderophore production, all the glassware were first soaked in 2N HCl solution for 24 h to avoid contamination of iron from the glassware. The chrome azurol S (CAS) solution was prepared by dissolving 60.5 mg dehydrated chrome azurol S in 50 ml double distilled water and further mixing with 10 ml of iron solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 10 mM HCl). This was then slowly added with 40 ml aqueous solution containing 72.9 mg cetyl trimethyl ammonium bromide with continuous stirring and the final solution was autoclaved.

The King's B agar was prepared using PIPES buffer (30.2 g) and Difco agar (18.0 g) and the pH was adjusted to 6.8 by addition of 0.1 N NaOH before autoclaving (Schwyn and Neilands, 1987). After cooling, the CAS solution (100 ml) was added along the wall of flask with gentle agitation to mix without formation of foam. The CAS agar thus prepared was poured in to the plates. After solidification, the plates were kept in the refrigerator ( $4^\circ\text{C}$ ) for 24 h. The overnight cultures of fluorescent pseudomonads (10  $\mu\text{l}$  each) were spotted on these CAS agar plates and incubated at  $28 \pm 2^\circ\text{C}$  for 48 h.

Formation of orange coloured zone around the colony was taken as positive for the siderophore production. The diameter of orange colored zone was recorded.

### 3.5.4 HCN production

The ability of the antagonistic methylbacteria to produce HCN were assessed as per the method of the method of Wei *et al.* (1991). Whatmann filter pads were placed on the lid of the petriplates and the plates sterilized. CAS medium amended with glycine (4.4 g/litre) was sterilized and poured into the sterile plates. The antagonists were streaked on the medium. The filter paper padding in each plate was soaked with two ml sterile picric acid solution. The plates were sealed with parafilm in order to contain gaseous metabolite produced by the antagonists and to allow for a chemical reaction with picric acid on the top.

After incubation for a weeks at 30°C, the colour change of filter paper was noted and the HCN production potential of the antagonists was assessed as per the following scoring.

No colour change	: Nil
Brownish colour change	: Weak
Brownish to orange	: Moderate
Completely orange	: Strong

## 3.6 Pot culture experiment

A pot culture experiment was conducted to assess the effect of five selected methylotrophs on growth and yield of *Coleus forskohlii* at the Medicinal and Aromatic plant unit, Department of Horticulture, Saidapur Farm, Dharwad during 2012 as described below.

### 3.6.1 Preparation of pots

The medium black soil collected from Saidapur Farm, MARS, University of Agricultural Sciences, Dharwad was filled in to earthen pots of 30 cm diameter at the rate of 8 kg per pot and used.

### 3.6.2 Preparation of standard 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  $28 \pm 2^{\circ}\text{C}$  for five days to get a population of  $10^9$  cells  $\text{ml}^{-1}$  of liquid culture. The culture broth was then mixed with sterilized lignite powder at 1:3 ratio.

### 3.6.3 Preparation of planting material

From the plants of *Coleus forskohlii* grown at Saidapur Farm, MARS, UAS Dharwad, cuttings were brought, trimmed to uniform size and used in the current investigation.

### 3.6.4 Cutting dip

One kg carrier based bioinoculant of PPFM was mixed with 15 litres of water and a slurry prepared. The stem cuttings were dipped in this slurry for 30 min and then planted in pots. In all, there were 7 treatments, with ten replications. Date of planting : 12-01-2012.

Treatment details

T <sub>1</sub>	PPFM 14
T <sub>2</sub>	PPFM 32
T <sub>3</sub>	PPFM 36
T <sub>4</sub>	PPFM 50
T <sub>5</sub>	PPFM 138
T <sub>6</sub>	Reference strain ( <i>Methylobacterium extorquens</i> )
T <sub>7</sub>	UIC

UIC : Un inoculated control.

### 3.6.5 Fertilizer application

The recommended dose of fertilizers for *Coleus forskohlii* is 40:60:50 kg NPK per hectare. N in the form of urea, P in the form of single superphosphate and K in the form of muriate of potash were applied to soil in pots 15 days after planting and mixed thoroughly. Watering was done thrice a week so as to maintain 70 per cent water holding capacity.

### 3.6.6 Phyllosphere spray with native PPFM bacterial isolates

The PPFM cultures were grown for 5 days ( $1 \times 10^9$  CFU ml<sup>-1</sup>) and diluted at 1:1 ratio with sterilized distilled water 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 45, 90 and 135 days after transplanting.

### 3.6.7 Plant growth parameters

The plant height, number of primary branches, leaf area, stem girth and chlorophyll content were recorded at 30, 60, 90 and 120 days interval.

#### 3.6.7.1 Plant height (cm)

The height of the plant was measured from the base to the growing tip of the shoot in cm at different stages of plant growth and the average worked out.

#### 3.6.7.2 Number of leaves per plant

The number of fully opened leaves was counted from the randomly selected plants and the mean was worked out.

#### 3.6.7.3 Number of branches per plant

Total number of branches per plant were counted from each pot and the mean was worked out.

#### 3.6.7.4 Leaf area (cm<sup>2</sup>)

The leaf area per plant was recorded from the five randomly selected plants. Leaf area was recorded by using disc method.

#### 3.6.7.5 Estimation of chlorophyll

The chlorophyll content was determined using single photoelectric analyzing diode (SPAD) instrument.

### 3.6.9 Harvesting

Harvesting was done at 135 days after planting, by uprooting plants from pots without disturbing roots using excess water. Date of harvesting: 26-05-2012

#### 3.6.9.1 Number of tubers per plant

Total number of tuberous roots were counted from each of five selected plants and expressed as mean number of tuberous roots per plant of fresh and dry weight of shoot biomass total weight of shoot and root biomass was recorded using a chemical balance.

#### 3.6.9.2 Fresh weight of tubers per plant (g/plant)

The fresh weight of the tuberous roots per plant and then weight was recorded by using a balance.

#### 3.6.9.3 Dry weight of tubers per plant (g/plant)

After taking fresh weight, the tuberous roots were sun dried and then dried in hot air over at 65-70 °C till the constant weight was reached and later the mean dry weight per plant was recorded.

#### 3.6.9.4 Dry matter content

The root (tubers) portions of the uprooted plants were separated and oven dried at 60°C to constant weight. The dry weights were then recorded separately for roots and average of the ten plants were expressed in grams per plant.

## 3.7 Estimation of soil enzymes

Rhizosphere soil samples were drawn from each treatment and used to estimate activities of different enzymes.

### 3.7.1 Dehydrogenase activity

Dehydrogenase activity in the soil samples was determined by following the procedure as described by Casida *et al.* (1964). Ten grams of soil and 0.2 g CaCO<sub>3</sub> were thoroughly mixed and dispensed in test tubes. To each tube, one ml of 3 per cent aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC), one ml of one per cent glucose solution and eight ml of distilled water were added, which was sufficient to leave a thin film of water above the soil layer. The tubes were stoppered with rubber cork and incubated at 30°C for 24 hrs. At the end of incubation, the contents of the tube were rinsed down into a small beaker and a slurry was made by adding 10 ml methanol. The slurry was filtered through Whatman No. 50 filter paper. Repeated rinsing of soil with one ml methanol was continued till the filtrate ran free of red color. The filtrate was pooled and made upto 50 ml with methanol in a volumetric flask. The intensity of red color was measured at 485 nm against a methanol blank using spectrophotometer. The concentration of formazan in soil samples were determined by reference to a standard curve prepared by using graded concentration of formazan. The results were expressed as µg of triphenyl formazan (TPF) formed g<sup>-1</sup> soil per day.

### 3.7.2 Phosphatase activity

Phosphatase activity of soil samples was determined by following the procedure of Evazi and Tabatabai (1979).

One gram of soil sample was placed in 50 ml Erlenmeyer flask to which 0.2 ml toluene followed by four ml of modified universal buffer (pH 7.5) were added. One ml of para-nitrophenol phosphate solution made in modified universal buffer was added to the flasks and contents of the flasks were mixed by swirling for 2 minutes. The flasks were stoppered and incubated at 37°C for one hour. After incubation, one ml of 0.5 M CaCl<sub>2</sub> and four ml of 0.5 M NaOH were added to the flask, swirled and filtered through Whatman No. 42 filter paper. The intensity of yellow colour developed was measured at 420 nm against the reagent blank using spectrophotometer.

Controls were maintained for each soil sample and were analyzed by following the same procedure described above except that the para nitrophenol phosphate solution was added after the addition of 0.5 M CaCl<sub>2</sub> and 0.5 M NaOH and just before filtration. The phosphatase activity in the soil samples was expressed as µg para nitrophenol formed per gram soil per hour with reference to the standard curve prepared by using graded concentrations of p-nitrophenol phosphate.

### 3.7.3 Urease activity

The procedure adopted to determine the urease activity of soil was essentially the same as adopted by Pancholy and Rice (1973) except the ammonia liberated due to hydrolysis of urea in the reaction mixture was determined by nesslerization as described by Jackson (1973).

Ten grams each of freshly collected soil samples were placed in 100 ml capacity Erlenmeyer flasks to which one ml toluene was added and allowed to stand for 15 minutes to permit complete penetration into soil. Each of these flasks was added with 10 ml of phosphate buffer (pH 6.7) and 10 ml of 10 per cent urea solution. For control flasks, urea solution was replaced by equal quantity of distilled water. The contents of the flask were well shaken for five minutes and incubated at 30°C for 24 hrs. After incubation, the contents of the flasks were filtered through Whatman No. 42 filter paper. The remaining soil in the flask was added with 15 ml of 1 N KCl solution shaken for five minutes and filtered. The volume of the total filtrate was made up to 100 ml in the volumetric flask using distilled water.

The amount of ammonia present in the filtrate was determined by nesslerization. One ml filtrate of each sample was transferred to 20 ml volumetric flask to which two ml of ten per cent sodium tartarate solution and 0.5 ml of Nessler's reagent were added. The volume was made up to 20 ml with distilled water.

The yellow color developed after 30 minutes was measured at 410 nm using a Spectrophotometer against the reagent blank.

The results obtained were expressed as  $\mu\text{g}$  of ammonia liberated per gram soil (oven dry basis) per day with reference to a standard curve outlined by using graded concentrations (0-100  $\mu\text{g ml}^{-1}$ ) of  $(\text{NH}_4)_2\text{SO}_4$  solution and developing the colour by nesslerization.

### 3.8 Population dynamics of PPFM in phyllosphere and rhizosphere of *Coleus forskohlii*

Rhizosphere soil samples were drawn from each treatment and used to estimate the population of PPFM. Similarly, leaf samples were collected and used to enumerate PPFM. One gram of leaf sample was ground using a pestle and mortar, serially diluted up to  $10^{-6}$  dilutions and one ml of the aliquot from  $10^{-6}$  was transferred to the sterile petridishes. The AMS medium was sterilized and poured into petridishes in triplicates. The plates were incubated at  $28 \pm 2^\circ\text{C}$ . The characteristic pink colonies growing over the medium were counted and expressed as  $10^{-6}$  cfu  $\text{g}^{-1}$  fresh weight of leaf tissue. Similarly, population count of mycotrophs from rhizosphere soil was determined and expressed as  $10^{-3}$  cfu  $\text{g}^{-1}$  dry weight of soil.

### 3.9 Statistical analysis of the data

The data were subjected to Completely Randomized Design (CRD) analysis as described by (Yates, 1937). The level of significance used in 'F' and 'T' test was  $P=0.01$ . Critical difference values were calculated whenever the 'F' test values are significant.

## 4. EXPERIMENTAL RESULTS

Methylotrophs were isolated from *Coleus* grown in different locations such as Nandihalli, Naganahatti, Rajahamsagada, Tarihall Hidakal of Belgaum district, Dharwad and Bangalore (GKVK). The results pertaining to the isolation, purification and characterization of methylotrophs, screening of *Methylobacterium* isolates for beneficial characters and also their effects on *C. forskohlii* plant under pot culture conditions are presented in this chapter.

### 4.1 Isolation of methylotrophs from *Coleus forskohlii*

Pink pigmented facultative methylobacteria (PPFM) were isolated from surfaces of fresh leaf samples (Plate 2), tubers, stem (Plate 3), roots (Plate 4) and tubers and root endophytes (Plate 5) as well as rhizosphere soils (Plate 6) of *C. forskohlii* plants collected from different places as shown in Table 1, Plate 1.

As many as 50 isolates were isolated, purified and given code numbers as PPFM series and maintained as the culture bank in the Department of Agricultural Microbiology, UAS, Dharwad.

### 4.2 Morphological characterization

The results of the morphological characterization of PPFM isolates are presented in Table 2. All the isolates observed were gram negative, rod shaped with the dimension ranging from 0.6- 1.0 x 1.0-1.5  $\mu\text{m}$ . Colonies were pink, pale pink or dark pink in color due to pigmentation (Table 2).

### 4.3 Biochemical characterization of methylotroph isolates

All the isolates were tested for different biochemical tests. All the isolates showed positive results for oxidase test, urease test, citrate and catalase test (Table 3).

#### 4.3.1 Utilization of different carbon sources

They were tested for utilization of different carbon compounds. Methylotrophs are able to grow on a wide variety of multi carbon compounds other than single carbon compounds. All the isolates showed positive results for different carbon sources viz., glucose, glycerol, tartarate and acetate (Table 4).

### 4.4 Testing of *Methylobacterium* isolates for production of growth promoting substances and other beneficial characters

*Methylobacterium* isolates were tested for production of indole acetic acid, gibberellic acid, P solubilization, siderophore and HCN production activities.

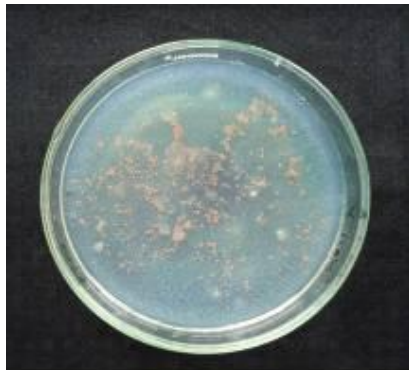
#### 4.4.1 Indole-acetic acid (IAA) production by *Methylobacterium* isolates

All the isolates were tested for indole acetic acid production. The production of indole acetic acid varied with the isolates, and the results are presented in Table 5.

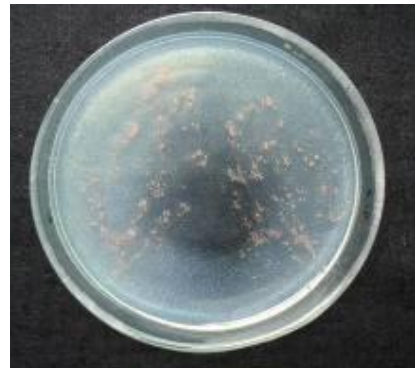
The highest indole acetic acid production was recorded in PPFM 36 and PPFM 50 (29.97  $\mu\text{g ml}^{-1}$  of culture filtrate) which was followed by PPFM 14 (29.83  $\mu\text{g ml}^{-1}$ ), while the reference strain, *Methylobacterium extorquens* produced (29.10  $\mu\text{g ml}^{-1}$ ). The lowest indole acetic acid production was recorded by PPFM 2 (22.47  $\mu\text{g ml}^{-1}$  of culture filtrate).

#### 4.4.2 Gibberellic acid production by *Methylobacterium* isolates

All the isolates showed positive results for gibberellic acid production and the results are presented in Table 5. The maximum GA production of 59.13  $\mu\text{g ml}^{-1}$  of culture filtrate was observed in PPFM 14 followed by PPFM 32 (59.06  $\mu\text{g ml}^{-1}$  of culture filtrate). The reference strain, *Methylobacterium extorquens* 45.06  $\mu\text{g ml}^{-1}$  of culture filtrate. The lowest GA production was observed in PPFM 8 (53.10  $\mu\text{g ml}^{-1}$  of culture filtrate).



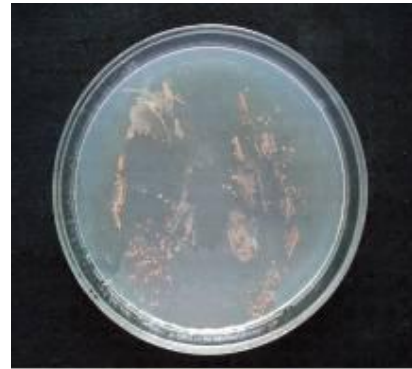
**Naganahatti-1**



**Rajahamsagada-1**



**Naganahatti-2**

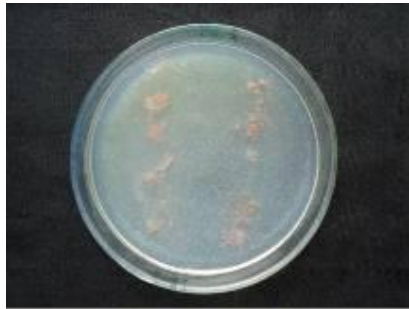


**Hidakaldam organic**



**Hidakaldam general1**

**Plate 2. Isolation of Prem by leaf imprinting method from different sources.**



Stem Dharwad-2

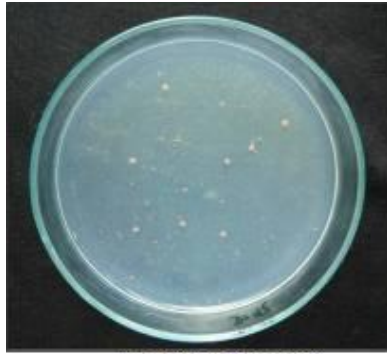


Naganahatti-3 stem

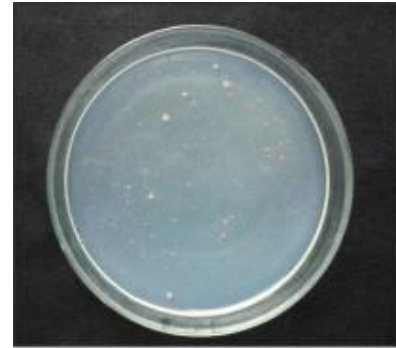
Plate 3. Isolation of PPFM from from stem surface of *Coleus forskohlii*



Plate 4. Isolation of PRFM from Rhizoplane of *Coleus forskohlii*

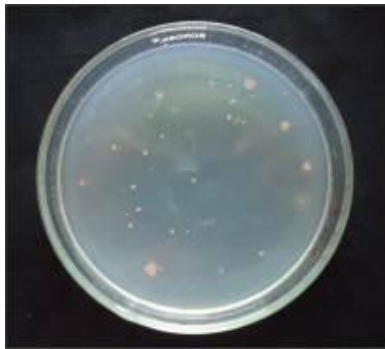


Endophyte Dharwad-1



Endophytes Hidakal

**Plate 5. Isolation of Prfm from root endophytes of *Coleus forskohlii***



Dharwad soil



Soil Rajhamsagada

**Plate 6. Isolation of Prfm from rhizosphere soil of *Coleus forskohlii***

**Table 2: Morphological characterization and Gram reaction of PPFM isolates**

Sl. No.	Code no. of the isolate	Cell size (µm)	Cell shape	Gram reaction	Pigmentation
1	PPFM 1	0.6x1.0	Rod	Negative	Dark pink
2	PPFM 2	0.8x1.2	Rod	Negative	Pink
3	PPFM 5	0.7 x 1.3	Rod	Negative	Light pink
4	PPFM 8	0.9 x 1.3	Rod	Negative	Light pink
5	PPFM 9	0.9x1.1	Rod	Negative	Dark pink
6	PPFM 10	0.5x1.0	Rod	Negative	Light pink
7	PPFM 14	0.7x1.1	Rod	Negative	Light pink
8	PPFM 15	1.0 x 1.2	Rod	Negative	Dark pink
9	PPFM 16	0.9x1.0	Rod	Negative	Light pink
10	PPFM 17	0.8 x 1.4	Rod	Negative	Pink
11	PPFM 20	0.6x1.2	Rod	Negative	Pink
12	PPFM 22	1.0x1.5	Rod	Negative	Pink
13	PPFM 24	0.8x1.5	Rod	Negative	Pink
14	PPFM 26	0.6x1.4	Rod	Negative	Light pink
15	PPFM 27	0.7x1.2	Rod	Negative	Pink
16	PPFM 28	0.9x1.5	Rod	Negative	Dark pink
17	PPFM 30	0.6x1.2	Rod	Negative	Light pink
18	PPFM 32	0.8x1.6	Rod	Negative	Light pink
19	PPFM 36	0.9x1.1	Rod	Negative	Pink
20	PPFM 37	0.9x1.5	Rod	Negative	Dark pink
21	PPFM 38	1.0x1.3	Rod	Negative	Light pink
22	PPFM 39	0.8x1.0	Rod	Negative	Pink
23	PPFM 40	0.3x1.1	Rod	Negative	Light pink
24	PPFM 41	0.5x1.2	Rod	Negative	Light pink
25	PPFM 42	0.7x1.0	Rod	Negative	Pink
26	PPFM 45	0.6x1.5	Rod	Negative	Dark pink
27	PPFM 49	0.6x1.3	Rod	Negative	Light pink
28	PPFM 50	0.6x1.0	Rod	Negative	Dark pink
29	PPFM 54	0.8x1.2	Rod	Negative	Light pink
30	PPFM 58	0.8x1.3	Rod	Negative	Pink
31	PPFM 59	0.8x1.0	Rod	Negative	Dark pink
32	PPFM 60	0.7x1.3	Rod	Negative	Light pink
33	PPFM 81	0.7x1.0	Rod	Negative	Dark pink
34	PPFM 83	0.6x1.0	Rod	Negative	Light pink
35	PPFM 100	0.6x1.2	Rod	Negative	Pink
36	PPFM 117	0.6x1.0	Rod	Negative	Dark pink
37	PPFM 119	0.8x1.2	Rod	Negative	Dark pink
38	PPFM 120	0.8x1.3	Rod	Negative	Light pink
39	PPFM 132	0.6x1.4	Rod	Negative	Light pink
40	PPFM 138	0.6x1.5	Rod	Negative	Light pink
41	PPFM 148	0.8x1.6	Rod	Negative	Dark pink
42	PPFM 151	0.9x1.2	Rod	Negative	Light pink
43	PPFM 156	0.9x1.1	Rod	Negative	Pink
44	PPFM 162	0.9x1.6	Rod	Negative	Pink
45	PPFM 165	0.9x1.3	Rod	Negative	Pink
46	PPFM 168	0.7x1.3	Rod	Negative	Pink
47	PPFM 170	0.7x1.2	Rod	Negative	Pink
48	PPFM 184	0.8x1.0	Rod	Negative	Pink
49	PPFM 188	0.6x1.5	Rod	Negative	Pink
50	PPFM 189	0.7x1.0	Rod	Negative	Pink

**Table 3: Biochemical characterization of PPFM isolates**

Sl. No.	Code no. of the isolate	Citrate utilization	Urease	Catalase	Oxidase
1	PPFM 1	+	+	+	+
2	PPFM 2	+	+	+	+
3	PPFM 5	+	+	+	+
4	PPFM 8	+	+	+	+
5	PPFM 9	+	+	+	+
6	PPFM 10	+	+	+	+
7	PPFM 14	+	+	+	+
8	PPFM 15	+	+	+	+
9	PPFM 16	+	+	+	+
10	PPFM 17	+	+	+	+
11	PPFM 20	+	+	+	+
12	PPFM 22	+	+	+	+
13	PPFM 24	+	+	+	+
14	PPFM 26	+	+	+	+
15	PPFM 27	+	+	+	+
16	PPFM 28	+	+	+	+
17	PPFM 30	+	+	+	+
18	PPFM 32	+	+	+	+
19	PPFM 36	+	+	+	+
20	PPFM 37	+	+	+	+
21	PPFM 38	+	+	+	+
22	PPFM 39	+	+	+	+
23	PPFM 40	+	+	+	+
24	PPFM 41	+	+	+	+
25	PPFM 42	+	+	+	+
26	PPFM 45	+	+	+	+
27	PPFM 49	+	+	+	+
28	PPFM 50	+	+	+	+
29	PPFM 54	+	+	+	+
30	PPFM 58	+	+	+	+
31	PPFM 59	+	+	+	+
32	PPFM 60	+	+	+	+
33	PPFM 81	+	+	+	+
34	PPFM 83	+	+	+	+
35	PPFM 100	+	+	+	+
36	PPFM 117	+	+	+	+
37	PPFM 119	+	+	+	+
38	PPFM 120	+	+	+	+
39	PPFM 132	+	+	+	+
40	PPFM 138	+	+	+	+
41	PPFM 148	+	+	+	+
42	PPFM 151	+	+	+	+
43	PPFM 156	+	+	+	+
44	PPFM 162	+	+	+	+
45	PPFM 165	+	+	+	+
46	PPFM 168	+	+	+	+
47	PPFM 170	+	+	+	+
48	PPFM 184	+	+	+	+
49	PPFM 188	+	+	+	+
50	PPFM 189	+	+	+	+

Note : + = activity

#### 4.4.3 P-solubilization by *Methylobacterium* isolates

All the fifty strains were tested for MPS activity on Pikovskaya's agar medium. The zone of solubilization by the strains was recorded on 8th day of incubation on Pikovskaya's medium. The diameter of the solubilization zones on Pikovskaya's agar medium by the PPFM isolates ranged from 2.10 to 13.95 mm (Plate 7). Among the strains, PPFM 14 significantly showed the largest zone of 13.95 mm, on Pikovskaya's agar medium. The growth of all isolates on Pikovskaya's agar medium occurred, zone of solubilization was not clear in many isolates. Hence, all the isolates grown in liquid medium and Pi release was quantified.

The amount of Pi released from TCP in Pikovskaya's broth by PPFM isolates was studied at 10 days after incubation (DAI) (Table 5). Among all the isolates, PPFM 138 was found to release the highest amount of Pi (10.07%) followed by PPFM 50 (9.80%). However, PPFM 20, PPFM26, and PPFM 81 released the lowest amount of Pi (8.07%).

#### 4.4.4 Siderophore production by *Methylobacterium* isolates

None of the isolates produced siderophores when tested on CAS medium (Table 5).

#### 4.4.5 HCN production by *Methylobacterium* isolates

None of the tested isolates produced HCN (Table 5).

#### 4.4.6 Antifungal activities of methylo-trophs isolates

The antifungal activities of all PPFM isolates was assessed against three important fungal pathogens namely *Sclerotium rolfsii*, *Fusarium Oxysporum* and *Rhizoctonia bataticola* following dual culture method (Table 6).

Eight out of fifty isolates inhibited *Sclerotium rolfsii* on potato dextrose agar medium. PPFM 37 produced the highest zone of inhibition (10.25 mm dia) and it is significantly different from other isolates. The reference strain *Methylobacterium extorquens* produced the zone of inhibition of 5.25 mm. The least zone of inhibition was observed with PPFM 42 and PPFM 54 (4.25 mm dia) each (Table 6, Fig. 1, Plate 8).

Ten isolates out of fifty inhibited the pathogen, *Fusarium oxysporum* on potato dextrose agar medium. PPFM 32 produced the highest zone of inhibition (14.25 mm dia) which was significantly different from other isolates. *Methylobacterium extorquens* strain produced zone of inhibition (8.25 mm). The least zone of inhibition was observed with PPFM 50 and PPFM 138 which produced 5.25 mm dia.

Nine isolates inhibited the pathogen *Rhizoctonia bataticola* on potato dextrose agar medium. PPFM 138 significantly produced the highest zone of inhibition (6.25 mm dia). The least zone of inhibition was observed with PPFM 14 which produced 2.35 mm dia.

#### 4.4.7 Antibacterial activities of methylo-troph isolates

The antibacterial activities of methylo-trophic isolates against four important bacterial pathogens namely *Xanthomonas oxanopodis*, *Ralstonia solanacearum*, *Xanthomonas oxanopodis* pv. *citri* and *Xanthomonas oxanopodium* pv. *Punicae* were assessed following the dual culture assay. But, unfortunately none of the isolates including reference strain did not inhibit any of the pathogens (Table 7).

### 4.5 Pot culture evaluation of the effect of selected methylo-trophic isolates on growth and tuber yield of *Coleus forskohlii*

Five efficient PPFM isolates were evaluated on *Coleus forskohlii* under pot culture condition (Plate 9). To study their influence on different plant parameters, tuber yield, population dynamics of PPFM in phyllosphere and rhizosphere and enzymatic activities in the rhizosphere.

**Table 4 Ability of PPFM isolates to utilize different carbon sources**

Sl. No.	Code no. of the isolate	Glucose	Glycerol	Tartarate	Acetate
1	PPFM 1	+	+	+	+
2	PPFM 2	+	+	+	+
3	PPFM 5	+	+	+	+
4	PPFM 8	+	+	+	+
5	PPFM 9	+	+	+	+
6	PPFM 10	+	+	+	+
7	PPFM 14	+	+	+	+
8	PPFM 15	+	+	+	+
9	PPFM 16	+	+	+	+
10	PPFM 17	+	+	+	+
11	PPFM 20	+	+	+	+
12	PPFM 22	+	+	+	+
13	PPFM 24	+	+	+	+
14	PPFM 26	+	+	+	+
15	PPFM 27	+	+	+	+
16	PPFM 28	+	+	+	+
17	PPFM 30	+	+	+	+
18	PPFM 32	+	+	+	+
19	PPFM 36	+	+	+	+
20	PPFM 37	+	+	+	+
21	PPFM 38	+	+	+	+
22	PPFM 39	+	+	+	+
23	PPFM 40	+	+	+	+
24	PPFM 41	+	+	+	+
25	PPFM 42	+	+	+	+
26	PPFM 45	+	+	+	+
27	PPFM 49	+	+	+	+
28	PPFM 50	+	+	+	+
29	PPFM 54	+	+	+	+
30	PPFM 58	+	+	+	+
31	PPFM 59	+	+	+	+
32	PPFM 60	+	+	+	+
33	PPFM 81	+	+	+	+
34	PPFM 83	+	+	+	+
35	PPFM 100	+	+	+	+
36	PPFM 117	+	+	+	+
37	PPFM 119	+	+	+	+
38	PPFM 120	+	+	+	+
39	PPFM 132	+	+	+	+
40	PPFM 138	+	+	+	+
41	PPFM 148	+	+	+	+
42	PPFM 151	+	+	+	+
43	PPFM 156	+	+	+	+
44	PPFM 162	+	+	+	+
45	PPFM 165	+	+	+	+
46	PPFM 168	+	+	+	+
47	PPFM 170	+	+	+	+
48	PPFM 184	+	+	+	+
49	PPFM 188	+	+	+	+
50	PPFM 189	+	+	+	+

Note : + = growth

**Table 5: Production of phytohormones, siderophores, HCN and Pi released by PPFM isolates**

Sl. No.	Code no. of the Isolates	IAA ( $\mu\text{g/ml}$ )	GA( $\mu\text{g/ml}$ )	Pi release (%)	Siderophore production	HCN production
1	PPFM 1	29.50 <sup>a</sup>	56.30 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
2	PPFM 2	22.47 <sup>d</sup>	54.10 <sup>bc</sup>	8.17 <sup>cde</sup>	-	-
3	PPFM 5	27.50 <sup>abc</sup>	55.95 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
4	PPFM 8	28.33 <sup>abc</sup>	53.10 <sup>c</sup>	8.07 <sup>cde</sup>	-	-
5	PPFM 9	29.50 <sup>a</sup>	56.20 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
6	PPFM 10	28.33 <sup>abc</sup>	56.10 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
7	PPFM 14	29.83 <sup>a</sup>	59.13 <sup>a</sup>	8.33 <sup>cde</sup>	-	-
8	PPFM 15	27.47 <sup>abc</sup>	57.05 <sup>abc</sup>	8.07 <sup>cde</sup>	-	-
9	PPFM 16	29.47 <sup>a</sup>	55.77 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
10	PPFM 17	25.47 <sup>cd</sup>	57.98 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
11	PPFM 20	27.17 <sup>abc</sup>	56.05 <sup>abc</sup>	8.07 <sup>cde</sup>	-	-
12	PPFM 22	29.83 <sup>a</sup>	58.95 <sup>ab</sup>	8.17 <sup>cde</sup>	-	-
13	PPFM 24	28.47 <sup>abc</sup>	54.55 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
14	PPFM 26	26.17 <sup>bc</sup>	53.95 <sup>c</sup>	8.07 <sup>cde</sup>	-	-
15	PPFM 27	28.23 <sup>abc</sup>	53.75 <sup>c</sup>	8.33 <sup>cde</sup>	-	-
16	PPFM 28	29.83 <sup>a</sup>	55.95 <sup>abc</sup>	8.33 <sup>cde</sup>	-	-
17	PPFM 30	29.37 <sup>a</sup>	55.02 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
18	PPFM 32	29.80 <sup>a</sup>	59.06 <sup>ab</sup>	9.57 <sup>ab</sup>	-	-
19	PPFM 36	29.97 <sup>a</sup>	58.95 <sup>ab</sup>	9.07 <sup>abc</sup>	-	-
20	PPFM 37	28.17 <sup>abc</sup>	57.78 <sup>abc</sup>	8.33 <sup>cde</sup>	-	-
21	PPFM 38	28.50 <sup>ab</sup>	57.66 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
22	PPFM 39	27.47 <sup>abc</sup>	57.20 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
23	PPFM 40	28.50 <sup>ab</sup>	57.05 <sup>abc</sup>	8.23 <sup>cde</sup>	-	-
24	PPFM 41	28.17 <sup>abc</sup>	55.77 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
25	PPFM 42	29.67 <sup>a</sup>	57.98 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-

Note : - = no production

Contd...

26	PPFM 45	29.50 <sup>a</sup>	56.05 <sup>abc</sup>	8.17	-	-
27	PPFM 49	27.67 <sup>abc</sup>	58.02 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
28	PPFM 50	29.97 <sup>a</sup>	59.03 <sup>ab</sup>	9.80 <sup>a</sup>	-	-
29	PPFM 54	28.17 <sup>abc</sup>	53.95 <sup>c</sup>	8.17 <sup>cde</sup>	-	-
30	PPFM 58	27.73 <sup>abc</sup>	53.75 <sup>c</sup>	8.17 <sup>cde</sup>	-	-
31	PPFM 59	27.33 <sup>abc</sup>	57.66 <sup>abc</sup>	8.10 <sup>cde</sup>	-	-
32	PPFM 60	28.33 <sup>abc</sup>	54.55 <sup>abc</sup>	8.33 <sup>cde</sup>	-	-
33	PPFM 81	29.50 <sup>a</sup>	57.05 <sup>abc</sup>	8.07 <sup>cde</sup>	-	-
34	PPFM 83	29.90 <sup>a</sup>	55.77 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
35	PPFM 100	29.17 <sup>a</sup>	57.98 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
36	PPFM 117	29.17 <sup>a</sup>	56.05 <sup>abc</sup>	8.40 <sup>bcd</sup>	-	-
37	PPFM 119	29.50 <sup>a</sup>	58.95 <sup>ab</sup>	8.57 <sup>b<sup>c</sup></sup>	-	-
38	PPFM 120	29.83 <sup>a</sup>	54.55 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
39	PPFM 132	28.07 <sup>abc</sup>	53.95 <sup>c</sup>	8.17 <sup>cde</sup>	-	-
40	PPFM 138	29.50 <sup>a</sup>	59.06 <sup>ab</sup>	10.07 <sup>a</sup>	-	-
41	PPFM 148	28.50 <sup>ab</sup>	53.75 <sup>c</sup>	8.17 <sup>cde</sup>	-	-
42	PPFM 151	29.47 <sup>a</sup>	55.02 <sup>abc</sup>	7.23 <sup>de</sup>	-	-
43	PPFM 156	28.50 <sup>ab</sup>	55.95 <sup>abc</sup>	8.13 <sup>cde</sup>	-	-
44	PPFM 162	28.00 <sup>abc</sup>	58.02 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
45	PPFM 165	29.50 <sup>a</sup>	57.78 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
46	PPFM 168	27.50 <sup>abc</sup>	57.66 <sup>abc</sup>	8.10 <sup>cde</sup>	-	-
47	PPFM 170	29.50 <sup>a</sup>	59.13 <sup>a</sup>	8.27 <sup>cde</sup>	-	-
48	PPFM 184	26.23 <sup>bc</sup>	55.02 <sup>abc</sup>	9.57 <sup>ab</sup>	-	-
49	PPFM 188	27.97 <sup>abc</sup>	55.95 <sup>abc</sup>	7.17 <sup>e</sup>	-	-
50	PPFM 189	27.60 <sup>abc</sup>	58.02 <sup>abc</sup>	8.17 <sup>cde</sup>	-	-
51	Reference strain	29.10 <sup>ab</sup>	45.60 <sup>d</sup>	8.10 <sup>cde</sup>	-	-
<b>SEm ± CD (1%)</b>		0.81	1.34	0.29		
		3.01	4.96	1.10		

Note : = No production



PPFM 14



PPFM 16

PPFM 38

**Plate 7. P- solubilization activity of PPFM Isolates**

**Table 6: Antagonistic activity of the PPFM isolates against different plant pathogenic fungi**

Sl. No.	Code no. of the Isolates	<i>Sclerotium rolfsii</i> (ZOI in dia. mm)	<i>Fusarium oxysporum</i> (ZOI in dia. In mm)	<i>Rhizoctonia bataticola</i> (ZOI in dia. In mm)
1	PPFM 1	-	-	-
2	PPFM 2	-	-	-
3	PPFM 5	-	-	-
4	PPFM 8	-	-	-
5	PPFM 9	-	-	-
6	PPFM 10	-	-	-
7	PPFM 14	5.25 <sup>c</sup>	12.25 <sup>b</sup>	2.35 <sup>e</sup>
8	PPFM 15	-	-	-
9	PPFM 16	5.10 <sup>cd</sup>	-	-
10	PPFM 17	-	9.25 <sup>d</sup>	-
11	PPFM 20	-	8.25 <sup>e</sup>	-
12	PPFM 22	-	-	-
13	PPFM 24	-	-	-
14	PPFM 26	-	-	3.40 <sup>d</sup>
15	PPFM 27	-	-	-
16	PPFM 28	-	6.25 <sup>f</sup>	-
17	PPFM 30	-	-	-
18	PPFM 32	-	14.25 <sup>a</sup>	3.60 <sup>cd</sup>
19	PPFM 36	5.38 <sup>bc</sup>	11.25 <sup>c</sup>	-
20	PPFM 37	10.25 <sup>a</sup>	-	-
21	PPFM 38	5.25 <sup>c</sup>	-	3.25 <sup>d</sup>
22	PPFM 39	-	-	-
23	PPFM 40	-	-	-
24	PPFM 41	-	-	-
25	PPFM 42	4.25 <sup>d</sup>	-	-
26	PPFM 45	6.25 <sup>b</sup>	-	-
27	PPFM 49	-	-	3.35 <sup>d</sup>

Note : - = no activity

Contd...

28	PPFM 50	-	5.25 <sup>d</sup>	4.35 <sup>b</sup>
29	PPFM 54	4.25 <sup>d</sup>	-	3.25 <sup>d</sup>
30	PPFM 58	-	-	-
31	PPFM 59	-	-	-
32	PPFM 60	-	-	-
33	PPFM 81	-	-	4.25 <sup>bc</sup>
34	PPFM 83	-	-	-
35	PPFM 100	-	-	-
36	PPFM 117	-	-	-
37	PPFM 119	-	-	-
38	PPFM 120	-	-	-
39	PPFM 132	-	-	-
40	PPFM 138	-	9.25 <sup>d</sup>	6.25 <sup>a</sup>
41	PPFM 148	-	-	-
42	PPFM 151	-	-	-
43	PPFM 156	-	-	-
44	PPFM 162	-	-	-
45	PPFM 165	-	-	-
46	PPFM 168	-	-	-
47	PPFM 170	-	-	-
48	PPFM 184	-	5.25 <sup>e</sup>	3.55 <sup>cd</sup>
49	PPFM 188	-	-	-
50	PPFM 189	-	-	-
51	Reference strain	5.25 <sup>c</sup>	8.25 <sup>e</sup>	-
<b>SEm ± CD (1%)</b>		0.26	0.25	0.18
		1.17	1.15	0.83

Note : - = no activity

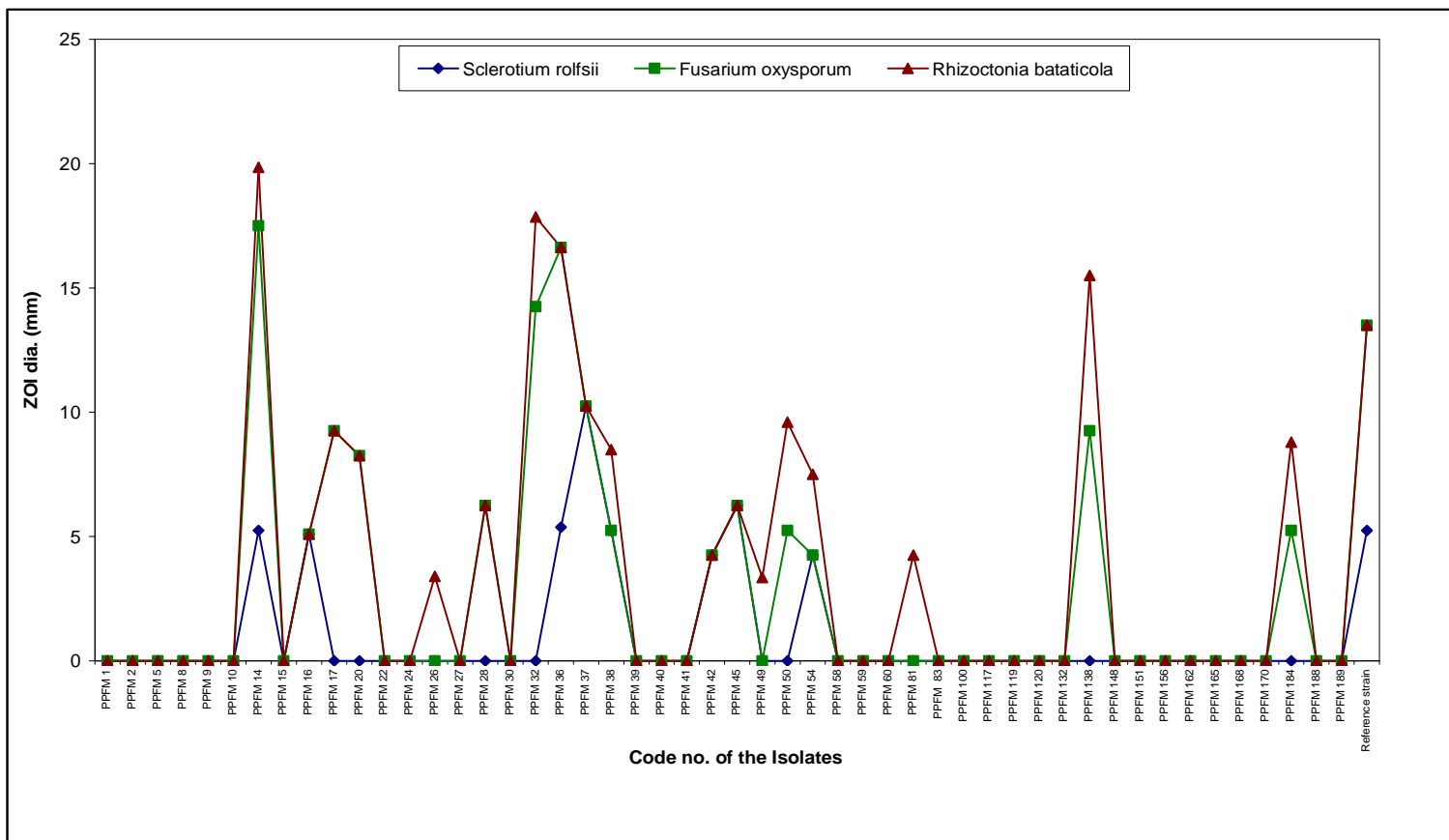
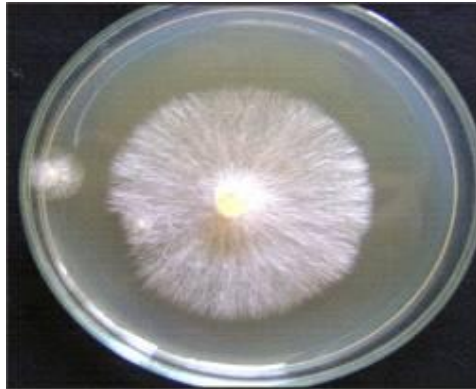


Fig. 1: Antagonistic activity of the PPFM isolates against different plant pathogenic fungi



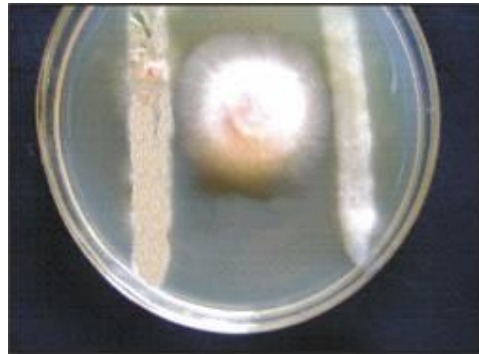
Control



PPFM 14



Control



PPFM 50



Control



PPFM 38

Plate 8. Antagonistic activity of PPFM isolates

#### 4.5.1 Plant height (cm)

The plant height of *Coleus forskohlii* on 30 DAP (days after planting) due to different PPFM isolates ranged from 13.69 to 20.94 (Table 9, Fig. 2, Plate 10, Plate 11). Among the different PPFM isolates, PPFM 50 recorded significantly maximum plant height (20.94 cm). The least height was recorded in PPFM 14 (13.69 cm). The reference strain recorded plant height (16.94 cm). The uninoculated control recorded (10.97 cm). At 60 DAP, PPFM 50 recorded maximum plant height (26.11 cm) and it was on par with PPFM 32, PPFM 36 and PPFM 138 isolates. The least was recorded in PPFM 14 (22.71 cm). The reference strain recorded plant height (23.11) and the uninoculated control recorded (17.88 cm). At 90 DAP, PPFM 50 recorded significantly higher plant height (30.74 cm) and it was on par with PPFM 31, PPFM 138 and reference strain. The least was recorded in PPFM 14 (27.40 cm). The reference strain recorded plant height (28.23) and the uninoculated control recorded (22.69 cm).

At 120 DAP, PPFM 50 recorded the maximum plant height (36.70 cm) which was on par with PPFM36 and was significantly different from other isolates. The least height was recorded in PPFM 14 (29.90 cm). The reference strain recorded plant height (28.75) and the uninoculated control recorded (24.85 cm). At harvest, PPFM 50 recorded maximum plant height (40.75 cm) and is significantly different from other isolates. The least was recorded in PPFM 14 (35.15 cm). The reference strain recorded plant height (31.88 cm) and the uninoculated control recorded (28.33 cm).

#### 4.5.2 Number of branches

There was no significant difference amongst different treatments both at 30 and 60 DAP (Table 9 and Fig 2). At 90 DAP, PPFM 50 recorded maximum number of branches (38) and it was significantly different from other isolates. The least was recorded in PPFM 14 (33.33). The reference strain recorded 28 number of branches. The uninoculated control recorded (24) branches.

At 120 DAP, PPFM 50 recorded maximum number of branches (57.00) and it was significantly different from other isolates. The least was recorded in PPFM 14 (45.00). The reference strain recorded number of branches (40.00). The uninoculated control recorded (34.00). And at harvest, PPFM 50 recorded maximum number of branches (57.67) and it was significantly different from other isolates. The least was recorded in PPFM 14 (46.67). The reference strain recorded number of branches (40.67). While the uninoculated control recorded (34.33).

#### 4.5.3 Number of leaves

The number of leaves of *Coleus forskohlii* at 30 DAP due to different PPFM isolates ranged from 15.70 to 22.20 (Table 9 and Fig 2). Among the different PPFM isolates, PPFM 50 recorded maximum number of leaves (22.20) and it was on par with PPFM 138, and significantly different from other isolates. The least was recorded in PPFM 14 (15.70). The reference strain recorded 18.90 number of leaves. The uninoculated control recorded (10.70). At 60 DAP, PPFM 50 recorded maximum number of leaves (71.60) and it was on par with PPFM 14, PPFM 32, PPFM 36, PPFM 138. The least was recorded in PPFM 14 (65.70). The reference strain recorded 67 leaves. The uninoculated control recorded (47). At 90 DAP, PPFM 50 recorded maximum number of leaves (127.90) and it was on par with PPFM 14, PPFM 32, PPFM 36, PPFM 138. The least no. of leaves was recorded in PPFM 14 (121.10). The reference strain recorded 115.20 number of leaves. The uninoculated control recorded (110.40).

At 120 DAP, PPFM 50 significantly recorded maximum number of leaves (179.00). The least was recorded in PPFM 14 (151.67). The reference strain recorded (150.00) number of leaves. The uninoculated control recorded (121.00). And, at harvest the number of leaves got reduced in all the treatments. PPFM 50 significantly recorded maximum number of leaves (147.00). The least amongst inoculated treatments was recorded in PPFM 14 (46.67). The reference strain recorded (40.67) number of leaves. The uninoculated control recorded (34.33).

**Table 7: Antagonistic activity of the PPFM isolates against different plant pathogenic bacteria**

Sl. No.	Code no. of the isolates	<i>Xanthomonas campestris</i>	<i>Ralstonia solanacearum</i>	<i>Xanthomonas oxanopodium pv. citri</i>	<i>Xanthomonas oxanopodium pv. punicae</i>
1	PPFM 1	-	-	-	-
2	PPFM 2	-	-	-	-
3	PPFM 5	-	-	-	-
4	PPFM 8	-	-	-	-
5	PPFM 9	-	-	-	-
6	PPFM 10	-	-	-	-
7	PPFM 14	-	-	-	-
8	PPFM 15	-	-	-	-
9	PPFM 16	-	-	-	-
10	PPFM 17	-	-	-	-
11	PPFM 20	-	-	-	-
12	PPFM 22	-	-	-	-
13	PPFM 24	-	-	-	-
14	PPFM 26	-	-	-	-
15	PPFM 27	-	-	-	-
16	PPFM 28	-	-	-	-
17	PPFM 30	-	-	-	-
18	PPFM 32	-	-	-	-
19	PPFM 36	-	-	-	-
20	PPFM 37	-	-	-	-
21	PPFM 38	-	-	-	-
22	PPFM 39	-	-	-	-
23	PPFM 40	-	-	-	-
24	PPFM 41	-	-	-	-
25	PPFM 42	-	-	-	-

Note : - = no activity

Contd...

26	PPFM 45	-	-	-	-
27	PPFM 49	-	-	-	-
28	PPFM 50	-	-	-	-
29	PPFM 54	-	-	-	-
30	PPFM 58	-	-	-	-
31	PPFM 59	-	-	-	-
32	PPFM 60	-	-	-	-
33	PPFM 81	-	-	-	-
34	PPFM 83	-	-	-	-
35	PPFM 100	-	-	-	-
36	PPFM 117	-	-	-	-
37	PPFM 119	-	-	-	-
38	PPFM 120	-	-	-	-
39	PPFM 132	-	-	-	-
40	PPFM 138	--	--	--	--
41	PPFM 148	-	-	-	-
42	PPFM 151	-	-	-	-
43	PPFM 156	-	-	-	-
44	PPFM 162	-	-	-	-
45	PPFM 165	-	-	-	-
46	PPFM 168	-	-	-	-
47	PPFM 170	-	-	-	-
48	PPFM 184	-	-	-	-
49	PPFM 188	-	-	-	-
50	PPFM 189	-	-	-	-
51	Reference strain	-	-	-	-

Note: - = No Activity



**Plate 9: General view of the pot experiment**

**Plate 9. General view of the pot Experiment**

**4.5.4 Leaf area (cm<sup>2</sup>)**

The leaf area of *Coleus forskohlii* at 30 DAP due to different PPFM isolates ranged from 429.99 to 445.60 (Table 9 and Fig 2). Amongst the different PPFM isolates, PPFM 50 significantly recorded maximum leaf area (445.60). The least amongst inoculated treatments was recorded in PPFM 14 (429.99). The reference strain recorded leaf area of (428.15). The uninoculated control recorded (410.43). At 60<sup>th</sup> DAP PPFM 50 significantly recorded maximum leaf area of (2844.33). The least was recorded in PPFM 14 (2532.07). The reference strain recorded leaf area of (2169.40). The uninoculated control recorded (1732.57). At 90 DAP, PPFM 50 significantly recorded maximum leaf area of (3570.17). The least leaf area recorded in PPFM 14 was 3268.98. The reference strain recorded leaf area of (3160.47). The uninoculated control recorded (2511.61).

At 120 DAP, PPFM 50 significantly recorded maximum leaf area (5861.69) and it was on par with PPFM 138. The least leaf area was recorded in PPFM 14 (5512.53). The reference strain recorded leaf area of (5433.63). The uninoculated control recorded (3784.54). And, at harvest PPFM 50 significantly recorded maximum leaf area (3010.13). The least was recorded in PPFM 14 (2632.07). The reference strain recorded leaf area of (40.67). The uninoculated control recorded (34.33).

**4.5.5 Stem girth (cm)**

The stem girth of *Coleus forskohlii* at 30 DAP due to different PPFM isolates ranged from 0.47 cm to 0.60cm (Table 9 and Fig 2). Amongst the different PPFM isolates, PPFM 50 recorded the maximum stem girth (0.60). The least was recorded in PPFM 14 (0.47). The reference strain recorded stem girth of (0.48). The uninoculated control recorded (0.43). At 60 DAP, PPFM 50 recorded maximum stem girth (0.88). The least was recorded in PPFM 14 and PPFM 138 (0.83) each. The reference strain recorded stem girth of (0.78). The uninoculated control recorded (0.66). At 90<sup>th</sup> DAP, PPFM 50 significantly recorded maximum stem girth (1.06). The least amongst inoculated treatments was recorded in PPFM 14 and PPFM 36 (1.87) and it was on par with PPFM 138 and reference strain. The reference strain recorded stem girth of (0.84). The uninoculated control recorded (0.65).

At 120 DAP, PPFM 50 significantly recorded maximum stem girth (1.38). The least was recorded in PPFM 14 (1.25). The reference strain recorded stem girth of (1.24). The uninoculated control recorded (1.04). And, at harvest, PPFM 50 recorded significantly maximum stem girth (1.42). The least was recorded in PPFM 14 (1.28). The reference strain recorded stem girth of (1.27). The uninoculated control recorded (1.10). +96

**Table 9: Influence of the selected PPFM isolates on growth parameters of *Coleus forskholii***

Code no. of the isolates	Plant height (cm/plant)					Chlorophyll content (SPAD values)				Stem girth (cm/plant)				
	30 DAP	60 DAP	90 DAP	120 DAP	At harvest (135 DAP)	30 DAP	60 DAP	90 DAP	120 DAP	30 DAP	60 DAP	90 DAP	120 DAP	At harvest (135 DAP)
PPFM 14	13.69 <sup>bc</sup>	22.71 <sup>ab</sup>	27.40 <sup>ab</sup>	29.90 <sup>cd</sup>	39.75 <sup>ab</sup>	35.87 <sup>a</sup>	40.22 <sup>a</sup>	43.19 <sup>ab</sup>	39.53 <sup>a</sup>	0.47 <sup>a</sup>	0.83 <sup>a</sup>	0.87 <sup>ab</sup>	1.25 <sup>a</sup>	1.28 <sup>ab</sup>
PPFM 32	15.29 <sup>abc</sup>	23.24 <sup>ab</sup>	28.22 <sup>a</sup>	31.75 <sup>bc</sup>	36.20 <sup>c</sup>	36.54 <sup>a</sup>	39.82 <sup>a</sup>	43.82 <sup>ab</sup>	39.50 <sup>a</sup>	0.54 <sup>a</sup>	0.87 <sup>a</sup>	0.91 <sup>ab</sup>	1.35 <sup>a</sup>	1.39 <sup>a</sup>
PPFM 36	16.44 <sup>abc</sup>	24.45 <sup>a</sup>	27.49 <sup>ab</sup>	35.25 <sup>a</sup>	35.15 <sup>c</sup>	36.06 <sup>a</sup>	40.33 <sup>a</sup>	45.81 <sup>a</sup>	42.23 <sup>a</sup>	0.51 <sup>a</sup>	0.84 <sup>a</sup>	0.87 <sup>ab</sup>	1.27 <sup>a</sup>	1.30 <sup>ab</sup>
PPFM 50	20.94 <sup>a</sup>	26.11 <sup>a</sup>	30.74 <sup>a</sup>	36.70 <sup>a</sup>	40.75 <sup>a</sup>	38.80 <sup>a</sup>	43.16 <sup>b</sup>	47.69 <sup>a</sup>	42.07 <sup>a</sup>	0.60 <sup>a</sup>	0.88 <sup>a</sup>	1.06 <sup>a</sup>	1.38 <sup>a</sup>	1.42 <sup>a</sup>
PPFM 138	15.76 <sup>abc</sup>	24.10 <sup>a</sup>	29.25 <sup>a</sup>	33.80 <sup>ab</sup>	38.50 <sup>b</sup>	38.78 <sup>a</sup>	41.52 <sup>a</sup>	44.59 <sup>a</sup>	43.07 <sup>a</sup>	0.52 <sup>a</sup>	0.83 <sup>a</sup>	0.91 <sup>ab</sup>	1.31 <sup>a</sup>	1.34 <sup>a</sup>
Reference strain ( <i>Methylobacterium extorquens</i> )	16.94 <sup>ab</sup>	23.11 <sup>ab</sup>	28.23 <sup>a</sup>	28.75 <sup>d</sup>	31.88 <sup>d</sup>	37.81 <sup>a</sup>	41.00 <sup>a</sup>	39.29 <sup>bc</sup>	43.00 <sup>a</sup>	0.48 <sup>a</sup>	0.78 <sup>a</sup>	0.84 <sup>ab</sup>	1.24 <sup>a</sup>	1.27 <sup>ab</sup>
UIC	10.97 <sup>c</sup>	17.88 <sup>b</sup>	22.69 <sup>b</sup>	24.85 <sup>e</sup>	28.33 <sup>e</sup>	35.75 <sup>a</sup>	39.04 <sup>a</sup>	36.69 <sup>c</sup>	41.13 <sup>a</sup>	0.43 <sup>a</sup>	0.66 <sup>a</sup>	0.65 <sup>b</sup>	1.04 <sup>a</sup>	1.10 <sup>a</sup>
SEm ±	0.81	0.83	0.76	0.72	0.48	0.88	0.89	0.72	1.05	0.02	0.02	0.01	0.04	0.01
CD(%)	3.03	3.10	2.87	3.57	2.39	NS	3.35	2.72	4.44	0.11	0.11	0.06	0.20	0.06

Note : DAP = days after planting  
UIC = Un inoculated control

Contd...

Code no. of the isolates	No. of leaves/plant					No. of branches/plant					Leaf area (cm <sup>2</sup> )/plant				
	30DAP	60 DAP	90 DAP	120 DAP	At harvest (135 DAP)	30 DAP	60 DAP	90 DAP	120 DAP	At harvest (135 DAP)	30 DAP	60 DAP	90 DAP	120 DAP	At harvest (135 DAP)
PPFM 14	15.70 <sup>ab</sup>	65.70 <sup>a</sup>	121.10 <sup>a</sup>	151.67 <sup>de</sup>	142.33 <sup>b</sup>	6.00 <sup>a</sup>	12.33 <sup>a</sup>	33.33 <sup>b</sup>	45.00 <sup>d</sup>	51.33 <sup>bc</sup>	429.99 <sup>c</sup>	2532.07 <sup>e</sup>	3268.98 <sup>d</sup>	5512.53 <sup>d</sup>	2632.07 <sup>e</sup>
PPFM 32	16.60 <sup>ab</sup>	65.90 <sup>a</sup>	123.60 <sup>a</sup>	156.00 <sup>c</sup>	137.33 <sup>c</sup>	6.67 <sup>a</sup>	13.33 <sup>a</sup>	35.00 <sup>ab</sup>	52.33 <sup>b</sup>	52.67 <sup>b</sup>	435.17 <sup>bc</sup>	2760.63 <sup>c</sup>	3327.35 <sup>c</sup>	5607.47 <sup>b</sup>	2809.60 <sup>c</sup>
PPFM 36	17.90 <sup>ab</sup>	69.10 <sup>a</sup>	123.60 <sup>a</sup>	154.00 <sup>cd</sup>	125.33 <sup>d</sup>	6.33 <sup>a</sup>	12.67 <sup>a</sup>	34.33 <sup>b</sup>	49.00 <sup>c</sup>	46.67 <sup>d</sup>	439.52 <sup>ab</sup>	2802.40 <sup>b</sup>	3259.07 <sup>e</sup>	5587.67 <sup>c</sup>	2932.30 <sup>b</sup>
PPFM 50	22.20 <sup>a</sup>	71.60 <sup>a</sup>	127.90 <sup>a</sup>	179.00 <sup>a</sup>	147.00 <sup>a</sup>	6.67 <sup>a</sup>	14.33 <sup>a</sup>	38.00 <sup>a</sup>	57.00 <sup>a</sup>	57.67 <sup>a</sup>	445.60 <sup>a</sup>	2844.33 <sup>a</sup>	3570.17 <sup>a</sup>	5861.69 <sup>a</sup>	3010.13 <sup>a</sup>
PPFM 138	21.00 <sup>a</sup>	68.70 <sup>a</sup>	122.90 <sup>a</sup>	159.67 <sup>b</sup>	125.33 <sup>d</sup>	6.67 <sup>a</sup>	13.00 <sup>a</sup>	34.67 <sup>ab</sup>	49.00 <sup>c</sup>	49.33 <sup>cd</sup>	432.71 <sup>bc</sup>	2537.63 <sup>d</sup>	3347.27 <sup>b</sup>	5849.73 <sup>a</sup>	2637.67 <sup>d</sup>
Reference strain ( <i>Methylobacterium extorquens</i> )	18.90 <sup>ab</sup>	67.10 <sup>a</sup>	115.20 <sup>a</sup>	150.00 <sup>e</sup>	117.00 <sup>e</sup>	6.33 <sup>a</sup>	13.67 <sup>a</sup>	28.00 <sup>c</sup>	40.00 <sup>e</sup>	40.67 <sup>e</sup>	428.15 <sup>c</sup>	2169.40 <sup>f</sup>	3160.47 <sup>f</sup>	5433.63 <sup>e</sup>	2269.40 <sup>f</sup>
UIC	10.70 <sup>b</sup>	47.00 <sup>b</sup>	110.40 <sup>a</sup>	121.00 <sup>f</sup>	114.67 <sup>e</sup>	5.33 <sup>a</sup>	10.67 <sup>a</sup>	24.00 <sup>d</sup>	34.00 <sup>f</sup>	34.33 <sup>f</sup>	410.43 <sup>d</sup>	1732.57 <sup>g</sup>	2511.61 <sup>g</sup>	3784.54 <sup>f</sup>	1932.63 <sup>g</sup>
SEm ±	1.24	1.80	3.14	0.68	1.08	0.58	1.15	0.82	0.77	0.67	2.02	0.82	1.00	0.76	0.89
CD(%)	4.68	6.75	11.79	2.86	4.56	NS	NS	3.44	3.23	2.81	8.50	3.47	4.22	3.22	3.73

Note: DAP= days after planting  
UIC = un inoculated control

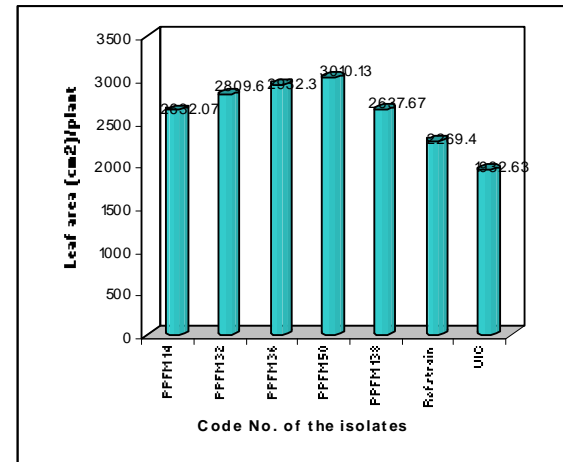
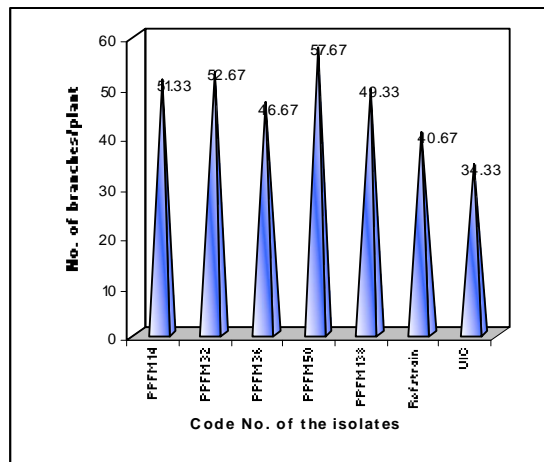
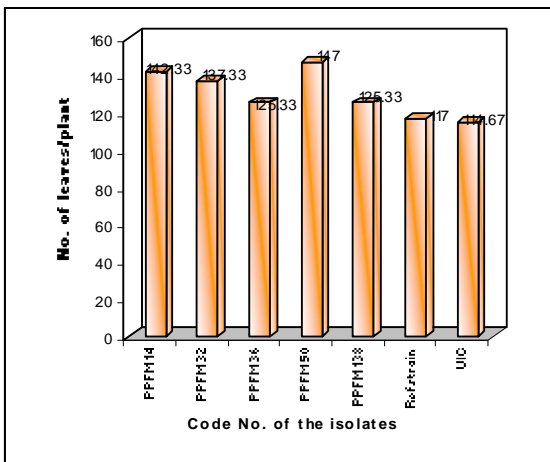
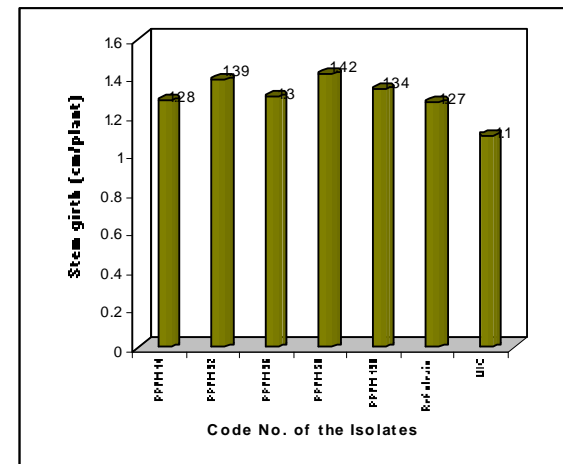
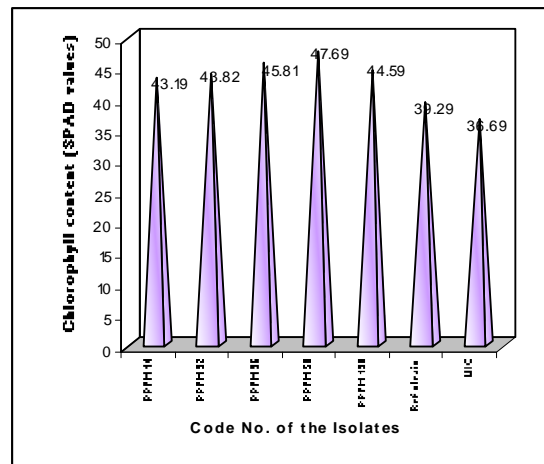
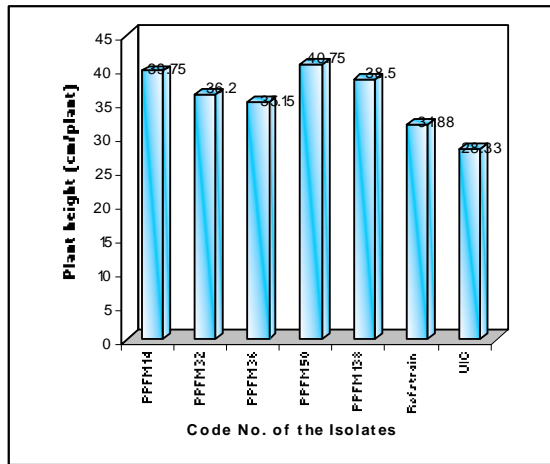


Fig. 2: Influence of the selected PPFM isolates on growth parameters of *Coleus forskholii*



Plate 10: Effect of inoculation of PPFM isolates on growth and biomass of *Coleus forskohlii*

### Plate 10 . Effect of inoculation of PPFM Isolates on growth and biomass of *Coleus forskohlii*

#### 4.5.6 Chlorophyll content

The chlorophyll content of *Coleus forskohlii* at 30 DAP due to different PPFM isolates ranged from 35.87 to 38.80 (Table 9 and Fig 2). Among the different PPFM isolates, PPFM 50 recorded maximum chlorophyll content (38.80) and it was on par with other isolates. The least was recorded in PPFM 14 (35.87). The reference strain recorded chlorophyll content (37.81). The uninoculated control recorded (35.75). At 60 DAP also PPFM 50 significantly recorded maximum chlorophyll content (45.12) and it was on par with other isolates. The least was recorded in PPFM 32 (39.82). The reference strain recorded chlorophyll content (41.00). The uninoculated control recorded (39.04). At 90 DAP, PPFM 50 recorded maximum chlorophyll content (47.69) and it was on par with PPFM 36 and PPFM 138. The least was recorded in PPFM 14 (43.19). The reference strain recorded chlorophyll content (39.29). The uninoculated control recorded (36.69).

At 120 DAP, the chlorophyll content of the plants was decreased. PPFM 50 recorded maximum chlorophyll content (42.07) and it was on par with other isolates. The least chlorophyll content was recorded in PPFM 32 (39.50). The reference strain recorded chlorophyll content of (43.00). The uninoculated control recorded (41.13).

## 4.6 Population dynamics of PPFM and soil enzyme activities

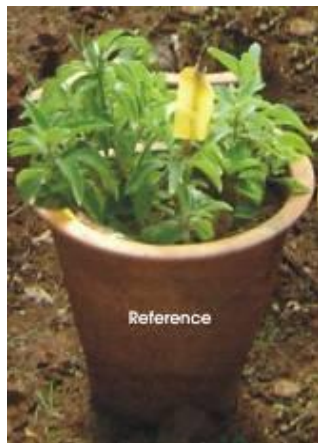
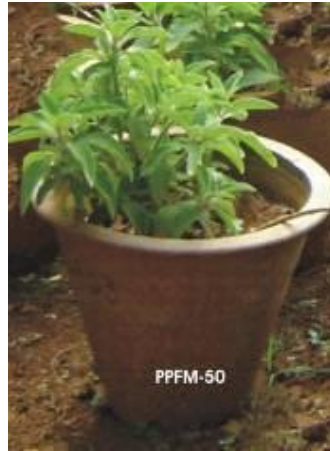
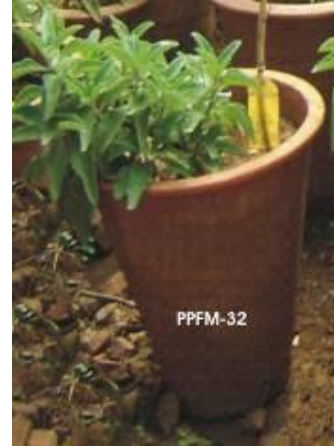
### 4.6.1 PPFM population in phyllosphere

Plants inoculated with PPFM isolates had significantly higher PPFM population in the phyllosphere than in the uninoculated plants at 90 DAP (Table 10 and Fig 3).

Inoculation of PPFM 50 recorded maximum population ( $103 \times 10^6$  cfu/g leaf) which was on par with PPFM 36 ( $101 \times 10^6$  cfu/g leaf). The lowest PPFM population was recorded in the treatment which received PPFM 14 ( $98 \times 10^6$  cfu/g leaf). The reference strain recorded ( $99.00 \times 10^6$  cfu/g leaf). The uninoculated control recorded ( $49 \times 10^6$  cfu/g leaf).

### 4.6.2 PPFM population in rhizosphere

Plants inoculated with PPFM 32 recorded the highest number of PPFM in the rhizosphere ( $25.50 \times 10^2$  cfu/g soil) which is significantly superior over other isolates and it was on par with PPFM 50 (Table 10 and Fig 3). The lowest PPFM population was recorded in the treatment which received PPFM 14. The reference strain recorded ( $18 \times 10^2$  cfu/g soil). The uninoculated control recorded ( $11.50 \times 10^2$  cfu/g soil). In general, population of PPFM was higher in the phyllosphere than in the rhizosphere.

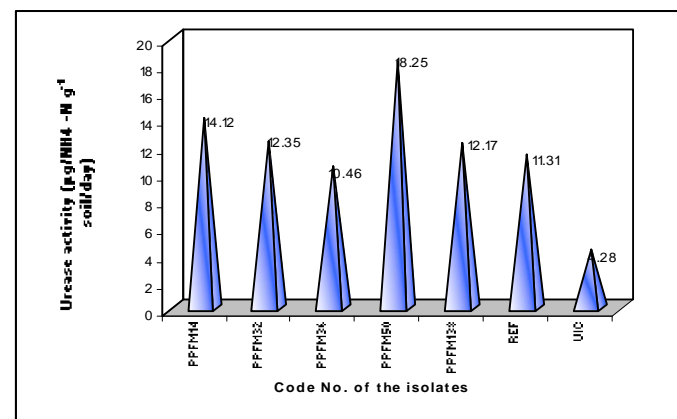
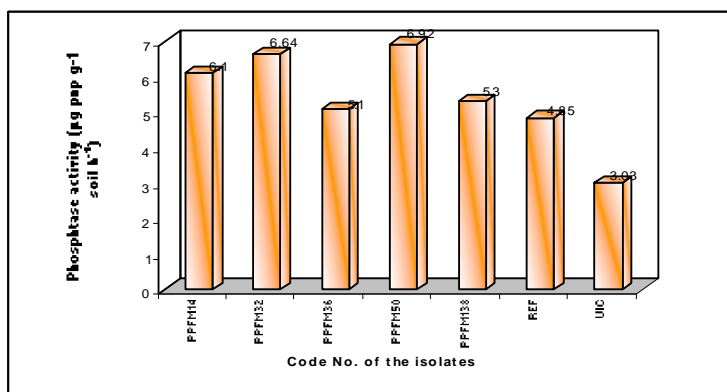
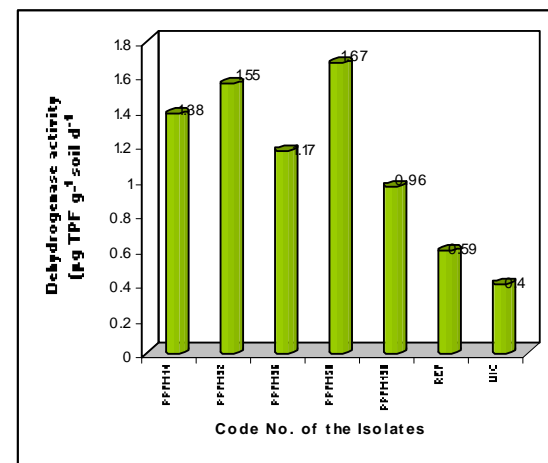
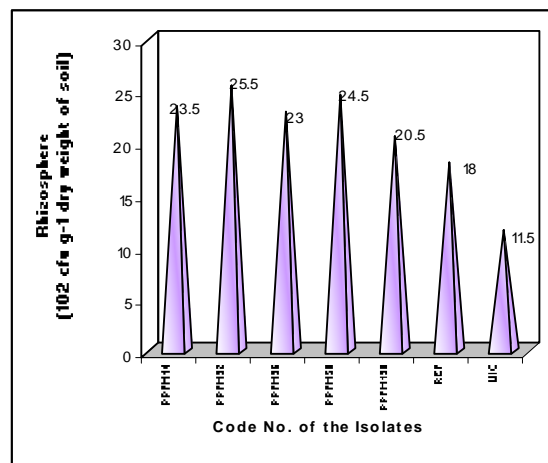
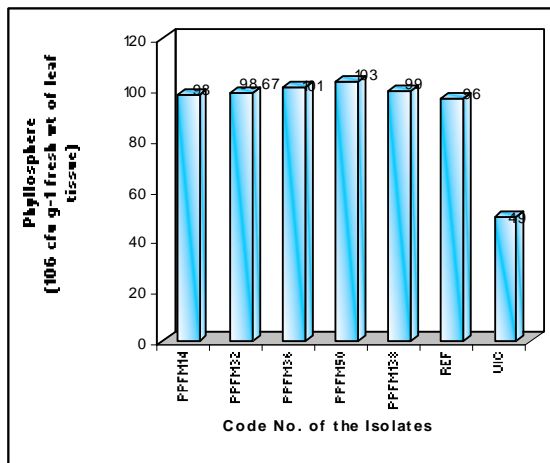


**Plate 11. Effect of inoculation of PPFM isolates on growth and biomass of *Coleus forskohlii* (Treatment wise)**

**Table 10: Phyllosphere and rhizosphere PPFM population and enzymatic activity as influenced by inoculation with selected PPFM isolates in *Coleus forskohlii***

Sl. No.	Code no. of the isolates	Phyllosphere (10 <sup>6</sup> cfu g <sup>-1</sup> fresh wt of leaf tissue)	Rhizosphere (10 <sup>2</sup> cfu g <sup>-1</sup> dry weight of soil)	Dehydrogenase activity (µg TPF g <sup>-1</sup> soil d <sup>-1</sup> )	Phosphatase activity (µg pnp g <sup>-1</sup> soil h <sup>-1</sup> )	Urease activity (µg/NH <sub>4</sub> -N g <sup>-1</sup> soil/d <sup>-1</sup> )
1	PPFM 14	98.00 <sup>cd</sup>	23.50 <sup>ab</sup>	1.38 <sup>b</sup> <sup>c</sup>	6.10 <sup>b</sup>	14.12 <sup>b</sup>
2	PPFM 32	98.67 <sup>c</sup>	25.50 <sup>a</sup>	1.55 <sup>ab</sup>	6.64 <sup>a</sup>	12.35 <sup>c</sup>
3	PPFM 36	101.00 <sup>ab</sup>	23.00 <sup>ab</sup>	1.17 <sup>cd</sup>	5.10 <sup>cd</sup>	10.46 <sup>d</sup>
4	PPFM 50	103.00 <sup>a</sup>	24.50 <sup>a</sup>	1.67 <sup>a</sup>	6.92 <sup>a</sup>	18.25 <sup>a</sup>
5	PPFM 138	99.00 <sup>b</sup> <sup>c</sup>	20.50 <sup>bc</sup>	0.96 <sup>d</sup>	5.30 <sup>c</sup>	12.17 <sup>c</sup>
6	Reference strain ( <i>methylobacterium extorquens</i> )	96.00 <sup>d</sup>	18.00 <sup>c</sup>	0.59 <sup>e</sup>	4.85 <sup>d</sup>	11.31 <sup>cd</sup>
7	UIC	49.00 <sup>e</sup>	11.50 <sup>d</sup>	0.40 <sup>e</sup>	3.03 <sup>e</sup>	4.28 <sup>e</sup>
	SEm±	0.55	0.87	0.02	0.09	0.30
	CD (1%)	2.31	4.29	0.08	0.43	1.46

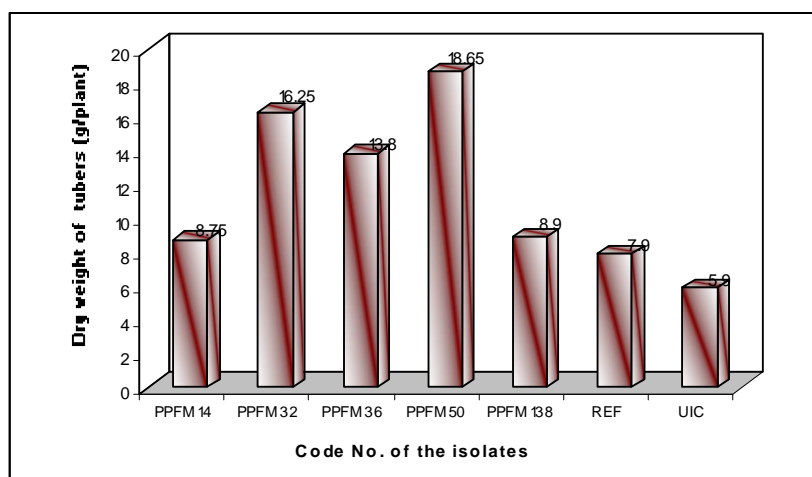
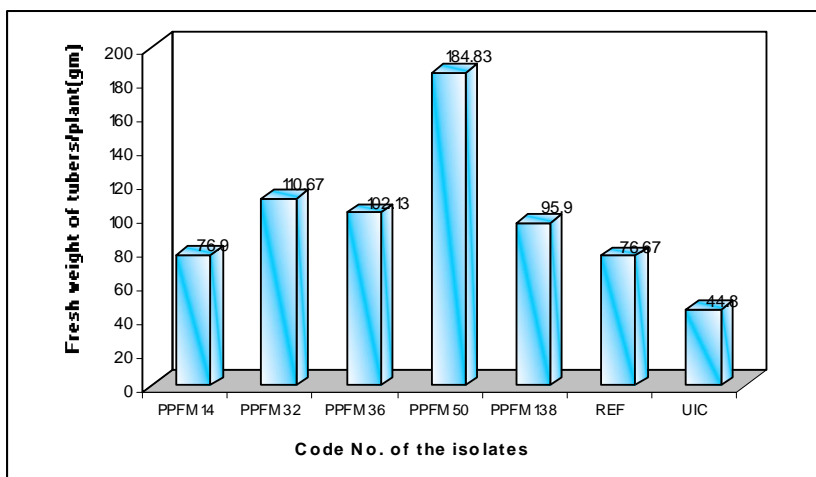
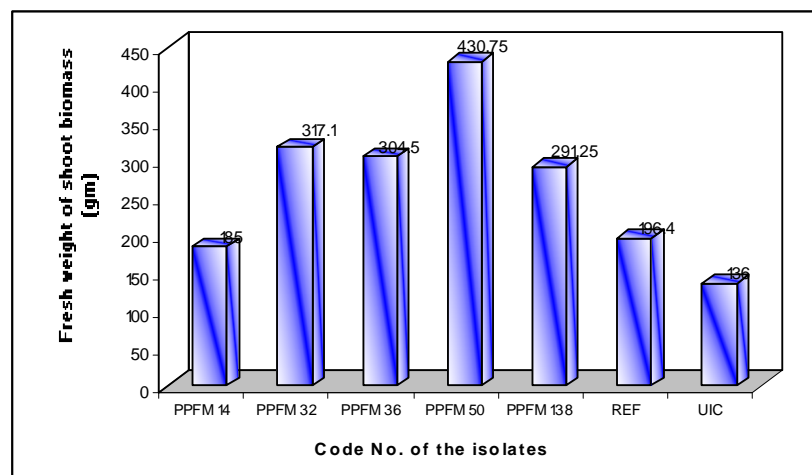
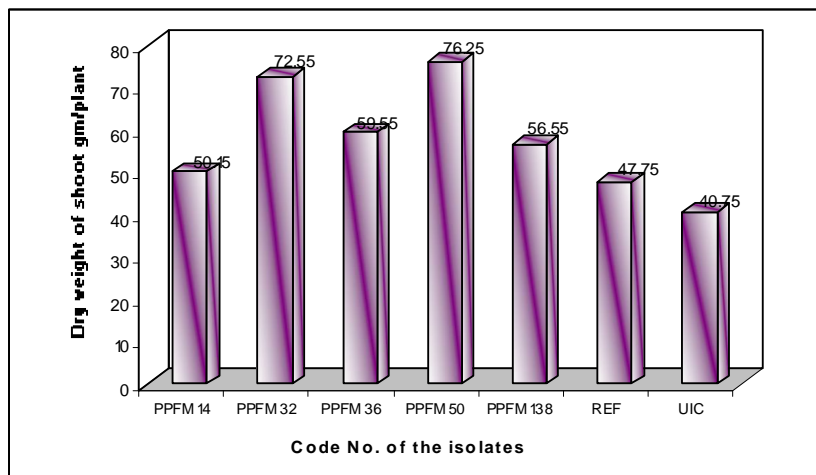
Note : UIC = uninoculated control



**Fig. 3: Phyllosphere and rhizosphere PPFM population and enzymatic activities as influenced by inoculation with selected PPFM isolates in *Coleus forskohlii***

**Table 11: Effect of selected PPFM isolates on plant biomass and tuber yield of *Coleus forskohlii***

Sl. No.	Code no. of the isolates	Dry weight of shoot g/plant	Fresh weight of Shoot biomass (g/plant)	No. of tubers/plant	Fresh weight of tubers (g/plant)	Dry weight of tubers (g/plant)
1	PPFM14	50.15 <sup>e</sup>	185.00 <sup>f</sup>	10.50 <sup>ef</sup>	76.90 <sup>c</sup>	8.75 <sup>de</sup>
2	PPFM32	72.55 <sup>b</sup>	317.10 <sup>b</sup>	18.50 <sup>b</sup>	110.67 <sup>b</sup>	16.25 <sup>b</sup>
3	PPFM36	59.55 <sup>c</sup>	304.50 <sup>c</sup>	15.50 <sup>c</sup>	102.13 <sup>bc</sup>	13.80 <sup>c</sup>
4	PPFM50	76.25 <sup>a</sup>	430.75 <sup>a</sup>	22.50 <sup>a</sup>	184.83 <sup>a</sup>	18.65 <sup>a</sup>
5	PPFM138	56.55 <sup>d</sup>	291.25 <sup>d</sup>	13.50 <sup>cd</sup>	95.90 <sup>bc</sup>	8.90 <sup>d</sup>
6	Reference strain ( <i>Methylobacterium extorquens</i> )	47.75 <sup>f</sup>	196.40 <sup>e</sup>	12.50 <sup>de</sup>	76.67 <sup>c</sup>	7.90 <sup>e</sup>
7	UIC	40.75 <sup>g</sup>	136.00 <sup>g</sup>	8.50 <sup>f</sup>	44.80 <sup>d</sup>	5.90 <sup>f</sup>
SEm±		0.34	0.21	1.80	0.50	7.00
CD (1%)		1.67	1.05	8.89	2.47	29.46



**Fig. 4: Effect of selected PPFM isolates on plant biomass and tuber yield of *Coleus forskohlii***



Plate 12. Effect of inoculation of PPFM isolates on root biomass of *Coleus forskohlii*



Plate 13. Effect of inoculation of PPFM isolates on root, growth and biomass of *Coleus forskohlii* (after harvest)

### 4.6.3 Dehydrogenase activity

The results of soil enzymatic activities at 90 DAP as influenced by inoculation with PPFM isolates are presented in Table 10 and Fig 3.

PPFM 50 showed the highest dehydrogenase activity (1.67  $\mu\text{g}$  TPF formed/g soil/day) which was significantly superior over rest of the isolates. The second highest was recorded in the treatment which received PPFM 32 (1.55  $\mu\text{g}$ ). The reference strain recorded (0.59 $\mu\text{g}$ ). The uninoculated control recorded (0.40  $\mu\text{g}$ ).

### 4.6.4 Urease

Significantly higher urease activity was observed in the treatment PPFM 50 (18.25  $\mu\text{g}$   $\text{NH}_4\text{-N/g}$  soil/day) followed by PPFM 14 (14.12  $\mu\text{g}$ ). The reference strain recorded (11.31 $\mu\text{g}$ ). The lowest urease activity was recorded in uninoculated control (4.28  $\mu\text{g}$ .) (Table 10 and Fig 3).

### 4.6.5 Phosphatase

Significantly increased phosphatase activity was recorded in the rhizosphere of *Coleus forskohlii* of PPFM 50 (6.92  $\mu\text{g}$  pnp/g soil/h) which is on par with PPFM 32 (6.64  $\mu\text{g}$  pnp/g soil/h) (Table 10 and Fig 3). Among the inoculations PPFM36 recorded the lowest phosphatase activity (5.10  $\mu\text{g}$  pnp/g soil/hr). Reference strain recorded (4.85  $\mu\text{g}$  pnp/g soil/hr). The uninoculated control recorded (3.03  $\mu\text{g}$  pnp/f soil/h).

## 4.7 Yield parameters and plant biomass production

### 4.7.1 Number of tubers

The data shows that number of tubers per plant as influenced by inoculation of PPFM were significantly higher as compared to uninoculated control (Table 11 and Fig 4). The highest number of tubers was observed in PPFM 50 (22.50) followed by PPFM 32 (18.50). The number recorded by reference strain was (13.50). The least number of tubers were recorded in PPFM 14 (10.50). The uninoculated control was recorded the least of all treatments (8.50).

### 4.7.2 Fresh weight of tuber (g)

The data shows that fresh weight of tubers per plant was improved significantly due to inoculation with PPFM as compared to control (Table 11). The highest fresh weight was observed in PPFM 50 (184.83) followed by PPFM 32 (110.67). The reference strain recorded (76.67). The least was recorded in PPFM 14 (76.90). The uninoculated control recorded 44.80 (Table 11, Fig 4 Plate 12).

### 4.7.3 Dry tuber yield (g/pl)

Significantly higher dry weight of tuber per plant was obtained with plants inoculated with PPFM as compared to control (Table 11). The highest dry weight was observed in PPFM 50 (18.65) followed by PPFM 32 (16.25). The reference strain recorded (7.90). The least was recorded in PPFM 14 (8.75). The uninoculated control recorded (5.90) (Table 11 and Fig 4) .

### 4.7.4 Fresh weight of shoot biomass (g/pl)

PPFM inoculation significantly increased shoot biomass as compared to control. The highest biomass was observed in PPFM 50 (430.75) followed by PPFM 32 (317.10). The reference strain recorded (196.40). The least amongst inoculated was recorded in PPFM 14 (185.00). The uninoculated control recorded (136.00) (Table 11, Fig 4, Plate 13).

### 4.7.5 Dry weight of shoot biomass (g/pl)

The data shows that dry weight of shoot biomass per plant due to inoculation with PPFM were significantly higher as compared to control. The highest biomass was observed in PPFM 50 (76.25) followed by PPFM 32 (72.55). The reference strain recorded (47.75). The least was recorded in PPFM 14 (50.15). The uninoculated control recorded (40.75) (Table 11 and Fig 4).

## 5. DISCUSSION

*Coleus forskohlii* is an important medicinal crop which contains forskohlin in their roots. The crop has a great potential in future due to the expected increase in demand for forskohlin which is widely used in the treatment of glaucoma, cardiac problems, eczema, asthma and hypertension and also certain types of cancers. Amongst various cultural practices conducive for enhancing root yield, nutrient management plays an important role in enhancing the yield per unit area. In the recent years, chemical fertilizers played a significant role in providing nutrients for intensive crop production, but increased use of chemical fertilizer in an imbalanced manner has created problems of multiple nutrient deficiencies, diminishing soil fertility and unsustainable crop yields. Therefore, to minimize their problems, there is a need to reduce use of chemical fertilizers and provide nutrients through other means. Therefore, emphasis is now focused on growing *Coleus* using organic manures, such as Farm Yard Manure (FYM) and biofertilizers like *Azotobacter*, *Azospirillum*, Phosphorus Solubilising Bacteria (PSB), Plant Growth Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhiza (AM fungi) which are ideal measures to produce roots of higher quality and safety.

The living space on the leaf surface, known as phyllosphere, shelters a great variety of microorganisms. The pink pigmented facultative methylotrophs of the genus *Methylobacterium* is one of the phyllosphere bacteria isolated from several plant species (Corpe and Rheem, 1989). It is well established that PPFM'S are colonizers of a wide array of crops and are capable of growing on C<sub>1</sub> compounds (formate, formaldehyde and methanol) as well as on a wide range of multicarbon substrates (Green and Bousfield 1983; Green 1992).

Recently, PPFM have emerged as a member of the Plant Growth Promoting phyllosphere bacterial group, enhancing growth and yields of crops. These bacteria secrete a variety of auxins (Ivanova *et al.*, 2001) and cytokinins (Lindstrom and Chistoserdova, 2002), which utilized by plants leading to an increased, germination, vigour index, growth, yield and systemic resistance (Madhaiyan *et al.*, 2005). They are ubiquitous in nature; found in a variety of habitats including phyllosphere, rhizosphere, root nodules, dust, fresh water and drinking water, lake sediments (Bousfield, 1985, Green and Bousfield, 1983 and Corpe and Rheem, 1989). The association of *Methylobacterium* sp. with plants seems to rely on a symbiotic relationship between the bacterium and host plants. The most common niche for the association between *Methylobacterium* and plant is the phyllosphere; where they utilize methanol, which is produced as a by product during cell wall synthesis inside leaves, and finally emitted through stomata. (Sy *et al.*, 2001).

The bioinoculants used as alternatives to chemical fertilizers usually target only the rhizosphere, and less attention has been paid to the phyllosphere. Although there are no prior reports on the beneficial effects of methylotrophic bacteria on the *Coleus* phyllosphere, Holland and Pollacco (1994) and Holland (1997) reported the beneficial effects of methylotrophic bacteria on various other crop plants.

Considering the importance of methylotrophs, the present investigation was attempted to study if one can extend the benefits to *Coleus* through isolation of native PPFM from *Coleus* grown in different locations and evaluating these isolates on the growth and tuber yield of *Coleus*.

In the present investigation, PPFM were isolated from various parts of *Coleus forskohlii* grown in different locations of the State. Isolations were made following leaf imprinting method using AMS medium which is a selective medium for isolating PPFM (Lindstrom and Chistoserdova, 2002). Various samples of leaves, roots, tuber and rhizosphere soil of *Coleus forskohlii* were used to isolate PPFM. Root endophytic methylotrophs were also isolated for the first time (18 in number). In all, as many as 50 methylotrophs were isolated and characterized.

The morphological characterization revealed that all methylotrophs were rods (0.6-1.0 x 1.0-1.5 µm) and gram negative with pigmentation ranging from dark to light pink color. PPFM isolates are easily identified by their pink color, which distinguishes them from other bacteria that are normally encountered on plant phyllosphere (Corpe and Basile, 1985).

The expression of pink pigmentation with varied level of intensity in PPFM indicates the presence of carotenoids (Fasim, 2003) which is known to protect their bacteria from intense high light and UV radiation (Liu *et al.*, 1993) and Patt *et al.* (1976) reported that *Methylobacterium* strains are rods which occur singly or occasionally in rosettes. They are often branched, pleomorphic, especially in stationary phase cultures. Methylo-troph isolates were further characterized by a series of biochemical tests. Thangamani and Sundaram (2005) reported that all *Methylobacterium* isolates were positive for citrate utilization, urease test, catalase activity, oxidase activity and indole production. They showed positive results for catalase test, urease test, oxidase and citrate utilization tests. Similarly, the isolates of the present investigation were found to be positive for the carbon utilization tests viz., glucose, glycerol, tartarate and acetate. Anthony (1986) also demonstrated that facultative methylo-trophs could utilize different carbon compounds and assimilate C1 compound via serine pathway.

The functional diversity of all the native PPFM isolates was analysed in terms of IAA and GA production, mineral phosphate solubilization (MPS) and antagonistic activities, in order to screen and select the most efficient PPFM isolates for further intervention studies with *Coleus* plants. In the present study, all the isolates produced IAA. The production of IAA varied with the isolates from 22.47  $\mu\text{g ml}^{-1}$  of culture filtrate to 29.97  $\mu\text{g ml}^{-1}$ . The highest IAA (29.97  $\mu\text{g ml}^{-1}$ .) produced by PPFM 36 and PPFM50 is comparable with that of the reference strain. IAA is known to stimulate division, extension and differentiation of plant cells, enhance root formation by promoting the conversion of parenchyma into xylem and phloem and regulate leaf area and fruit ripening (Muromtsev *et al.*, 1987).

Many epiphytic and soil microorganisms are able to synthesize and secrete auxins, primarily IAA (Felt *et al.*, 1987 and Libbert *et al.*, 1966) due to which they influence the growth of plants. The first report on the production of indole acetic acid in significant amount by four different methylo-trophs was reported by Ivanova *et al.* (2001). The production of plant growth regulators like auxins, zeatin and related cytokinins by methylo-trophs and their positive influence on seed germination and seedling growth has been reported earlier (Rangaswami and Bagyaraj, 1993 and Madhaiyan *et al.*, 2006). Jones (2010) observed variability among PPFM isolates in promoting IAA (0.14 to 25.12  $\mu\text{g/ml}$ ). Radha *et al.* (2009) documented production of IAA by PPFM ranging from 9.04  $\mu\text{g/ml}$  to 28.15  $\mu\text{g/ml}$ .

Gibberellic acid is another group of plant growth regulators, which act by modifying the plant morphology (Atzorn *et al.*, 1988). It is known to induce the uptake of minerals like K and Ca, increase the chlorophyll content, soluble sugars and protein content of plants. In the present study, all the isolates produced GA, which ranged from 53.10 (PPFM 5) to 59.13 (PPFM 14)  $\mu\text{g ml}^{-1}$  of culture filtrate. Their values were higher than the amount produced by the reference strain (45.6  $\mu\text{g ml}^{-1}$ ). Anu Rajan (2003) documented the production of gibberellic acid by eight of the PPFM isolates tested and the GA production varied from 10.9  $\mu\text{g ml}^{-1}$  to 106.97  $\mu\text{g ml}^{-1}$ . Jones (2010) reported that PPFM isolates produced GA ranging from 1.33 to 6.83  $\mu\text{g}/25\text{ml}$ .

Microorganisms are known to effectively recycle inorganic P in rhizosphere. Microbes which have the ability to release Pi from insoluble phosphate present in soil have been advocated to be used in biofertilization programme. PPFM have the ability to solubilize inorganic P (Jones, 2010). Hence, the PPFM isolates obtained in this study, were screened for their phosphate solubilizing ability. All the fifty isolates solubilized phosphorous ranging from 7.23 to 10.07 per cent. The highest Pi release was achieved by PPFM 138 (10.07%) which is higher than that of the reference strain (8.10%) indicating the superiority of the strain. The results obtained here reflects the possibility of using a single culture to provide both Plant Growth Promoting Substances (PGPS) and Pi in the rhizosphere of *Coleus*. The mineral phosphate solubilization has been reported for the first time by PPFM 117 isolates of PPFM from the Western Ghats (Jones *et al.*, 2007; Jones, 2010).

It was interesting to note that all the isolates produced IAA, GA and solubilised P also. In addition to beneficial traits like PGPS production and P- solubilization, antagonistic activity could be another important desirable property of the PPFM isolates to be used as microbial inoculants. In this context, all the isolates were examined for antifungal activities *in vitro* against *Sclerotium rolfsii*, *Rhizoctonia bataticola*, and *Fusarium oxysporum* which are important phytopathogenic fungi. Out of 50 isolates nine exhibited antifungal activity against *Sclerotium rolfsii*, 10 against *Fusarium oxysporum* and *Rhizoctonia bataticola*.

However, only one strain (PPFM 14) showed antagonistic activity against all the three pathogenic fungi indicating its superiority. PPFM 32, PPFM 36, PPFM 38, PPFM 50, PPFM 54, PPFM 138, PPFM 184 and reference strain showed activity against any two fungi. It was observed that none of the PPFM isolates exhibited antibacterial activity (Ganesan and Gnanamanikyam, 1987).

It was interesting to note that many PPFM isolates exhibited all the agronomically important traits such as PGPS production, MPS activity and antifungal activity, indicating their potential to be used in integrated nutrient and disease management of *Coleus* in an ecofriendly manner.

Hence, based on their functional characteristics, five efficient PPFM isolates were selected and evaluated for their influence on growth and tuber yield of *Coleus* (Table 8), under pot cultures. All the PPFM isolates significantly improved the growth parameters when compared to uninoculated control. Out of the isolates tested, PPFM 50 was the best isolate in all the parameters. It improved plant height, number of leaves, number of branches, chlorophyll content and stem girth when compared to uninoculated control. All these parameters were increased by 44, 48, 68, 30 and 29 percent respectively (Table 12). In fact this isolate was even better than the reference strain. Incidentally, PPFM 50 produced the highest quantity of IAA (29.97( $\mu\text{g/ml}$ )), GA (59.03( $\mu\text{g/ml}$ )) and solubilized P (9.80%) which have contributed to higher growth. Basile *et al.* (1969) first demonstrated the growth enhancing effects of PPFM on plants in a tissue culture system, where they produced vitamin B12 and stimulated the growth of a liverwort, *Scapania nemorosa*. Interestingly, they also produced growth hormones *viz.*, cytokinins (Koeing *et al.*, 2002), auxins (Ivanova *et al.*, 2001; Omer *et al.*, 2004) and biocontrol potential against *Sclerotium rolfsii* (Jones *et al.*, 2007).

Similar improvement in growth parameters due to inoculation with methylobacteria has been observed by Radha *et al.* (2009) in Soybean and Jones *et al.* (2007) in grapevine.

Shoot biomass and tuber yield of *Coleus* were also significantly increased due to PPFM inoculation. Again, out of 5 isolates tested, PPFM 50 showed the highest biomass and tuber yield. The percent increase in tuber yield due to inoculation with PPFM 50 was 216.10 against uninoculated control (136.07) against the reference strain (Table 13), thus indicating the superiority of the strain. The beneficial effect of PPFM on crop growth and yield has been studied by several workers in several crops including cotton (Madhaiyan *et al.*, 2005), rice (Senthilkumar, *et al.* 2002), groundnut (Sureshreddy, 2002), tomato (Thangamani and Sundaram, 2005), soybean, blackgram and sugarcane (Madhaiyan *et al.*, 2005). Suresh Reddy *et al.* (2002) studied the effect of combined inoculation of PPFMs and *Rhizobium* on groundnut cultivar Co(Gn)4 and observed that there was significant increase in plant growth, biomass production and yield parameters of groundnut. 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. Our findings are in confirmation with the results of Holland (1997) in soybean.

Studies on population dynamics of PPFM both in phyllosphere and rhizosphere and the enzymatic activities (dehydrogenase, urease and phosphatase) indicated significantly higher values in all the PPFM inoculated treatments when compared to UIC. Amongst the isolates, PPFM 50 resulted in the highest values of these parameters. Senthilkumar *et al.* (2002) and Madhaiyan (2002) recorded maximum PPFM population in the leaves followed by roots in greengram and soybean, respectively. The higher population of PPFM in the phyllosphere than in the rhizosphere could be due to the fact that they utilize the gaseous methanol that is emitted by plants through stomata as carbon and energy source and promote the growth of their host through the release of metabolites (Daniel *et al.*, 2006). The higher population of PPFM registered in rhizosphere of *Coleus* (due to inoculation with PPFM) has contributed to higher activity of all these soil enzymes. Inoculation and foliar application of PPFM increased nitrogenase, urease and phosphatase enzymes in the rhizosphere soils of rice, tomato and pepper when compared to uninoculated control (Madhaiyan *et al.*, 2009).

Thus, PPFM 50 with a very high PGPS production ability, MPS activity and antifungal activity turned out to be the best isolate and its inoculation resulted in doubling of the root yield when compared to un inoculated control (UIC), under pot cultures. However, the results need to be corroborated with field studies

**Table 8: Particulars of the selected PPFM isolates**

Sl. No.	Code no. of the isolate	sources	IAA ( $\mu\text{g/ml}$ )	GA( $\mu\text{g/ml}$ )	Pi release (%)	Antifungal activity		
						<i>Sclerotium rolfsii</i> (ZOI in dia. mm)	<i>Fusarium oxysporum</i> (ZOI in dia. mm)	<i>Rhizoctonia bataticola</i> (ZOI in dia. mm)
1	PPFM 14	Nandihalli	29.83	59.12	8.33	5.25	12.25	2.35
2	PPFM 32	Tarihaal	29.80	59.06	9.57	-	14.25	3.60
3	PPFM 36	Naganahatti	29.97	58.95	9.07	5.38	11.25	-
4	PPFM 50	Nandihalli	29.97	59.03	9.80	-	5.25	4.35
5	PPFM 138	Hidakal	29.50	59.06	10.07	-	9.25	6.25

**Table 12: Per cent increase in growth parameters by PPFM 50**

Sl. No.	Treatment	Plant height at harvest	No. of leaves at 120 days	No. of branches at harvest	Chlorophyll content at 90 DAP	Stem girth at harvest
1	Over UIC	43.84	47.90	67.98	29.98	29.09
2	Over strain reference ( <i>methylobacterium extorquens</i> )	27.82	19.33	41.79	21.37	11.81

**Table 13: Per cent increase in shoot biomass and tuber yield by PPFM 50**

<b>Sl. No.</b>	<b>Treatment</b>	<b>Fresh shoot biomass</b>	<b>Dry shoot biomass</b>	<b>Fresh tuber yield</b>	<b>Dry tuber yield</b>
1	Over UIC	216.72	87.11	212.56	216.10
2	Over reference ( <i>methylobacterium extorquens</i> )	119.32	59.68	141.07	136.07

## **Future line of work**

1. Field scale studies using efficient PPFM isolates
2. Effect of efficient PPFM isolates on quality of tuber of *Coleus*
3. Combination studies involving PPFM with other microbial inoculants
4. Effect of PPFM isolates on rooting in *Coleus* needs to be evaluated

## 6. SUMMARY AND CONCLUSIONS

An attempt was made to study the effect of methylotrophs, isolated from *Coleus* grown in different locations on the growth and yield of *Coleus forskohlii* under pot culture conditions.

1. As many as fifty pink pigmented facultative methylotrophs were isolated using different plant parts of *Coleus* on AMS medium, which is a selective medium for PPFMs.
2. The isolates were characterized based on the morphological and biochemical characteristics. All the isolates were gram negative, rod shaped and varied in cell size. All of the isolates were found to be positive for oxidase, urease, catalase and citrate production.
3. They were then screened for their beneficial traits such as production of plant growth promoting substances (IAA, GA) siderophore production, HCN production and antagonistic activity and phosphate solubilization. All the 50 PPFM isolates have shown negative results for siderophore and HCN production.
4. All the fifty isolates were tested for MPS activity and antagonistic activity against *Sclerotium rolfsii*, *rhizoctonia bataticola*, and *fusarium oxysporum*. The studies on functional characterization clearly revealed, that the 14 PPFM isolates possessed mineral phosphate solubilization activity and 19 PPFM isolates were antagonistic against *S. rolfsii*, *fusarium oxysporum* and *rhizoctonia bataticola*. Among the isolates PPFM138 significantly produced higher amount of Pi, while PPFM14 was superior in controlling all the three phytopathogens.
5. Based on the functional characterization and ability to inhibit the phytopathogens five isolates were selected and later, pot culture experiment was conducted on medicinal plant *Coleus forskohlii*. And the reference strain *Methylobacterium extorquens* was also used in the pot culture study.
6. Increase in the soil enzyme activities viz., dehydrogenase, phosphatase and urease were found to be significantly higher in PPFM treated *Coleus forskohlii* over uninoculated control.
7. The data on plant height, shoot biomass, chlorophyll content, stem girth, leaf area, and yield of tubers per plant as influenced by *Methylobacterium* isolates and application were recorded.
8. Out of five promising isolates and reference strain, PPFM 50 emerged the best organism in plant growth promotion and tuber yield of the *coleus forskohlii* plant.
9. Thus, the per cent increase in tuber yield due to inoculation with PPFM 50 is 216.10 against uninoculated control 136.07 against the reference strain, thus indicating the superiority of the strain.

Thus, the present study indicated that PPFM (50) was the most efficient organism in plant growth promotion and tuber yield of *coleus forskohlii* plant and it was superior compared with the reference strain (*Methylobacterium extorquens*).

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## Appendix I: Composition of media

### Ammonium mineral salt medium (AMS) (Whittenbury *et al.*, 1970)

NH <sub>4</sub> Cl	:	0.50 g
K <sub>2</sub> HPO <sub>4</sub>	:	0.70 g
KH <sub>2</sub> PO <sub>4</sub>	:	0.54 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	:	1.00 g
CaC <sub>12</sub> ·2H <sub>2</sub> O	:	0.20 g
FeSO <sub>4</sub> ·7H <sub>2</sub> O	:	4.00 mg
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	:	100.0 µg
MnCl <sub>4</sub> ·4H <sub>2</sub> O	:	30.0 µg
H <sub>3</sub> BO <sub>4</sub>	:	300.0 µg
CoC <sub>12</sub> ·6H <sub>2</sub> O	:	200.0 µg
CuC <sub>12</sub> ·2H <sub>2</sub> O	:	10.0 µg
NiC <sub>12</sub> ·6H <sub>2</sub> O	:	20.0 µg
Ha <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	:	60.0 µg
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	6.8
Methanol	:	0.5%

### Pikovskaya's medium (Pikovskaya, 1948)

Glucose	10 g
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	5 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5 g
Yeast extract	0.5 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g
NaCl	0.1 g
FeSO <sub>4</sub>	Traces
Distilled water	1000 ml
Agar	15 g
pH	6.8 – 7.0

**Czapadox mineral salt agar medium**

Sodium nitrate (NaNO <sub>3</sub> )	: 2.0 g
Potassium phosphate	: 1.0 g
Magnesium sulphate	: 0.5 g
Potassium chloride	: 0.5 g
Carboxymethyl cellulose	: 5.0 g
Peptone	: 2.0 g
Agar	: 20.0 g
Distilled water	: 1000 ml

**Nutrient agar**

Glucose	: 5.0g
Peptone	: 5.0g
Beef extract	: 3.0g
Sodium chloride	: 5.0g
Agar	: 15.0g
pH	: 7.0

**Simmon's citrate agar**

Sodium citrate	: 0.2g
Magnesium sulphate	: 0.02g
Ammonium dihydrogen phosphate	: 0.1g
Dipotassium hydrogen phosphate	: 0.1g
Sodium chloride	: 0.5g
Bromothymol blue	: 0.008g
Agar	: 2.0g
Distilled water	: 100.0ml
pH	: 6.8

**Trypticase soy agar medium**

Tryptone	: 17.0g
Soyatone	: 3.0g
Glucose	: 2.50g
Agar	: 18.0g
Distilled water	: 1000ml

## Appendix II: Composition of stains

### Gram Stain Solutions

#### Crystal violet solution

Crystal violet	-	10 g
Ammonium oxalate	-	4 g
Ethanol	-	100 ml
Distilled water	-	400 ml

#### Iodine solution

Iodine	-	1 g
Potassium iodide	-	2 g
Ethanol	-	25 ml
Distilled water	-	100 ml

#### Alcohol

Distilled water	-	5 ml
Ethanol	-	95 ml

#### Counter stain

2.5% Safranin in ethanol	-	10 ml
Distilled water	-	100 ml

### Preparation of IAA and GA standards

#### A. Hathway reagent

0.1N Ferric chloride	: 1 ml
0.1N HCl	: 1 ml
Distilled water	: 100 ml

Add 1 ml 0.1 M potassium ferricyanide to the above solution. Prepare fresh.

#### Sapler's reagent

0.5 M FeCl <sub>3</sub>	: 1.0 ml
35% HClO <sub>4</sub>	: 50 ml

#### Sukowsky's reagent

H <sub>2</sub> SO <sub>4</sub> (conc.)	: 150ml
Distilled water	: 250ml
FeCl <sub>3</sub> .6H <sub>2</sub> O (0.5M)	: 7.5ml

0.5M FeCl<sub>3</sub>: 0.81g FeCl<sub>3</sub> in 10ml H<sub>2</sub>O.

#### B. Reagent used for the estimation of GA (Spectrophotometric estimation)

- 1. Zinc acetate solution** : 21.9 g of zinc acetate was dissolved in 80 ml of distilled water. One ml of glacial acetate acid was added and the volume made upto 100 ml with distilled water.
- 2. Potassium ferrocyanide solution** : 1.6 g of potassium ferrocyanide solution in 100 ml of distilled water.

## Appendix III: Reagents used in soil enzyme studies

### Dehydrogenase activity

#### 2, 3, 5-Triphenyl tetrazolium chloride (TTC) solution (3%)

TTC	3 g
Distilled water	100 ml

#### TPF standard solution (500 $\mu$ g TPF ml<sup>-1</sup>)

TPF	50 mg
Methanol	80 ml
Final volume	100 ml

The standard curve drawn in the range of 10  $\mu$ g to 90  $\mu$ g TPF ml<sup>-1</sup>

### Phosphatase activity

SL. No	Reagent	Composition	
1	Modified universal buffer(MUB)	THAM	12.1g
		Maleic acid	11.6g
		citric acid	14.0g
		Boric acid	6.3 g in 488 ml of
		1NaOH	
		Final volume	1000 ml
2	p-Nitrophenyl phosphate solution(0.025M)	p-Nitrophenyl phosphate	0.42 g
		MUB	40 ml
		Final volume	50 ml
3	Standard p-Nitrophenol solution(1000 ppm)	p-Nitrophenol	1 g
		final volume	1000 ml

# ISOLATION AND CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHS (PPFM) FROM *Coleus forskohlii* AND THEIR INFLUENCE ON GROWTH AND TUBER YIELD

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## ABSTRACT

Investigations were carried to isolate Pink Pigmented Facultative Methylophs (PPFM), characterize them and study their beneficial effects on *Coleus forskohlii*. Samples of *C. forskohlii* were collected grown in different villages such as Nandihalli, Naganahatti, Rajahamsagada, Tarihalli, Hidakal, of Belgaum district, UAS, Dharwad and UAS, Bangalore (GKVK). As many as fifty isolates were isolated from these samples on selective AMS medium. The isolates were characterized based on their morphological and biochemical characteristics. The results revealed that all the isolates were Gram negative and rod shaped with the dimension ranging from 0.6-1.0 x 1.0-1.5  $\mu\text{m}$ . The colonies were pink in colour due to pigmentation. The biochemical characteristics revealed that all the isolates showed positive for oxidase, urease, citrate and catalase tests. All the isolates utilized glucose, glycerol, tartarate and acetate.

Further, the isolates were screened for beneficial characters. The results revealed that the highest indole acetic acid production was recorded in the isolates PPFM 36 and PPFM 50 (29.97  $\mu\text{g ml}^{-1}$  of each culture filtrate) and the maximum GA production (59.13  $\mu\text{g ml}^{-1}$  of culture filtrate) was by the isolate. The isolates were also tested for P-solubilization activity, antagonistic activity, siderophore and HCN production. The maximum Pi release was observed in PPFM 138 (10.07%). None of the isolates produced HCN and siderophores. And, as many as nineteen isolates showed antifungal activity against three important fungal pathogens of *Coleus* namely *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia bataticola*. Based on these beneficial characters, three efficient PPFM isolates were selected for the pot culture experiment. The results have revealed that all the PPFM isolates performed better than the reference strain (*Methylobacterium extorquens*) and uninoculated control. The isolate PPFM 50 isolated from Nandihalli village was most promising which recorded significantly higher growth and tuber yield parameters of *C. forskohlii* followed by PPFM 32.