STUDIES ON METHYLOTROPHS AND THEIR BENEFICIAL EFFECTS ON SOYBEAN (*Glycine max* (L.) Merrill)

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STUDIES ON METHYLOTROPHS AND THEIR BENEFICIAL EFFECTS ON SOYBEAN (*Glycine max* (L.) Merrill)

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By

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CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON METHYLOTROPHS AND THEIR BENEFICIAL EFFECTS ON SOYBEAN (*Glycine max* (L.) Merrill)**" *submitted by* **Miss RADHA T. K.,** *for the degree of* **MASTER OF SCIENCE (AGRICULTURE)** *in* **AGRICULTURAL MICROBIOLOGY** *to the University of Agricultural Sciences, Dharwad, is a record of research work carried out by her during the period of her study in this university, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.*

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Affectionately Dedicated

to

My Beloved

Parents & Sisters

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Introduction

I. INTRODUCTION

Plant microbe relationships like the soybean - *Bradyrhizobium japonicum* symbiosis or the genetic transformation of plant tissues of *Agrobacterium* sp. are physiologically meaningful to the plant (Holland and Polacco, 1994). Soil is a habitat for a vast, complex and interactive community of soil organisms, whose activities largely determine the chemical and physiological properties of the soil and growth of the plant. From seed germination until a plant reaches maturity, it lives in close association with soil organisms. The vast majority of plant associated soil organisms inhabit in the rhizosphere, defined as the zone around roots and bacterial growth is stimulated by the release of nutrients. Within the rhizosphere, there is a continuous interaction between plant roots and rhizosphere organisms. These interactions can have an important influence on plant growth.

Although rhizosphere appears to be too complex to allow its manipulation, specific bacteria can be applied to seed or roots, which cause an alteration in the composition of the rhizosphere. Such manipulations have important and exciting implications. In addition to the manipulation of the microflora and control disease causing organisms, it should be possible to promote the activity of beneficial ones, such as mycorrhizal fungi, associative nitrogen fixers, actinorhizae and *Rhizobium* spp. The plant canopy is also a host to wide array of microorganisms, which are beneficial, harmful and

neutralistic. 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 methylotroph *i.e.* (PPFM) and non-pigmented facultative methylotroph *i.e.* (NPFM) bacteria which are capable of growing on C_1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substrates such as C_2, C_3 and C_4 compounds. PPFMs are ubiquitous in nature and frequently reported on various plant species, those are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves. These bacteria are commonly found in soils, as well as on the surfaces of leaves, seeds and in the rhizosphere of a wide variety of plants, with highest numbers present on actively growing and meristamatic tissue, sometimes averaging 10^4-10^6 colony forming units (cfu) per leaflet (Dunleavy, 1988; Corpe, 1985; Corpe and Rheem, 1989; Hirano and Upper, 1992; Holland and Placco,

1992, 1994; Chanaprame *et al.*, 1996; Holland, 1997; Shepelyakovskaya *et al.*, 1999).

Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins (Dileepkumar and Dube, 1992; Holland and Placco 1992; 1994; Holland, 1997; Ivanova *et al.*, 2001; Omer *et al.*, 2004) and to alter agronomic traits like branching, seedling vigour, rooting and heat/cold tolerance (Holland, 1997; Freyermuth *et al.*, 1996).

Soybean (*Glycine max* (L.) Merrill) is a fascinating crop with innumerable possibilities of not only improving agriculture but also supporting industries. It occupies about 69.34 million hectares of area with 152.26 million tonnes of annual world production and productivity of 2.19 tonnes per hectare.

In India, it is grown over an area of 7.20 million hectares with a productivity of 944 kg per hectares (Anon., 2004). In Karnataka, it has occupied an area of 1.78 lakh hectares with the production of 1.36 lakh tonnes and a productivity of 876 kg per hectares (Anon., 2004). Being well suited to black soils of transitional tract of Karnataka and irrigated river belts of Krishna, it is a major crop of Belgaum, Dharwad, Haveri, Bidar and Bagalkot districts of the state. The golden bean contains the highest lysine rich protein (43.2%) among the

leguminous crop with 19.5 per cent of oil containing fairly high amount of unsaturated fatty acids (Gopalan *et al.*, 1994).

The productivity of soybean is often very much influenced by effective rhizosphere microflora. Based on these approaches, an attempt was made to study the effect of combined inoculation of growth promoting rhizobacteria such as *Methylobacterium* and *Rhizobium* on growth and yield of soybean with the following objectives.

1. Isolation, purification and characterization of methylotrophs from various sources predominantly from seed surface, rhizosphere and phyllosphere of different crops
2. Screening of methylotroph isolates for growth promoting substances and other beneficial properties
3. To study the effect of methylotrophs on growth and yield of soybean plants (pot culture studies)

Review of Literature

II. REVIEW OF LITERATURE

Increased yield response of crop plants have been observed following individual seed inoculation with *Rhizobium* (Dorosinky and Kayrov, 1975; Hernandez and Hill, 1983) and pink pigmented facultative methylotrophic bacteria (Holland and Polacco, 1994). Dual inoculation of legumes may have the potential to exploit synergistic effects on nitrogen fixation by Rhizobia accompanied by other organisms. The subject of dual inoculation of white clover with *Rhizobium* and VA mycorrhizal fungi was raised by Smith and Draft (1978). For a period it was considered that additional benefit from the mycorrhizae occurred only under the conditions of low fertility where the fungal hyphae scavenged plant nutrients otherwise unavailable to the host legume. Now it is well recognized that co-inoculation may have wider application (Badr-El-Din and Moawad, 1988) and other organisms like *Pseudomonas fluorescence* (Nishijima *et al.*, 1988) and *Bacillus thuringiensis* or indirectly by improving the health and vigour of the host like *Bacillus* as well have been reported to enhance nodulation directly like *Pseudomonas fluorescence* (Nishijima *et al.*, 1998) and *Bacillus thuringiensis* (Keyser and Lif, 1992).

When inoculated *Rhizobium* colonize the rhizosphere and enhance the plant growth by providing it with nitrogen (Kundu and Gaur, 1980). Whereas PPFMs colonize rhizosphsere as well as phyllosphere and enhance the plant growth by providing plant growth hormones like cytokinins and auxins (Ivanova *et al.*, 2000).

This chapter reviews the literature pertaining to the present study.

2.1 METHYLOTROPHS

Methylotrophic bacteria are ubiquitous in nature and frequently reported on various plant species, are a substantial part of the aerobic and heterotrophic microflora of the surfaces of young leaves. These bacteria are capable of growing on C_1 compounds like methanol and methylamine and also on a variety of C_2, C_3 and C_4 compounds. The genus *Methylobacterium* was originally reported as facultatively methane utilizing bacteria (Patt *et al.*, 1976). However, as the description of *Methylobacterium* excluded organisms that could not utilize methane, an amended genus name was proposed (Green and Bousifield, 1983) which would allow the inclusion of all PPFM strains, thus removing methane assimilation as an essential feature of the genus.

Bousifield and Green (1985) reclassified the genus *Protomonas* on the basis of the priority of the genus *Methylobacterium* over the genus *Protomonas*. *Protomonas extroquens* was renamed as *Methylobacterium extorquens*.

The genus *Methylobacterium* is composed of a variety of pink pigmented facultative methylotroph (PPFM) and non-pigmented facultative methylotroph (NPFM) bacteria which are capable of growing on C_1 compounds such as formate, formaldehyde, methanol and

methylamine as well as on a wide range of multicarbon growth substrates such as C_2, C_3 and C_4 compounds (Lidstrom, 1992).

Green and Bousifield (1982) studied the metabolism of C_1 compounds and the production of single cell protein from methanol, a large number of methanol utilizing bacteria were isolated from a wide variety of natural resources and the different species or different genera were assigned to the genus *Methylobacterium* on the basis of characteristics with minor differences. The numerical phonetic study of pink pigmented facultatively methylotrophic bacteria suggested the genus *Methylobacterium* is the appropriate place for these organisms.

Green and Bousifield (1982) examined 149 strains using 140 biochemical, physiological and morphological features, found that *Methylobacterium organophilum* strain xx to be phenotypically very similar to many methane non-utilizing, pink-pigmented, facultatively methylotrophic bacteria (PPFMs), *Methylobacterium organophilum* fell into either of two related clusters with >70 per cent similarity, which were well separated from other facultative methylotrophs and non-methylotrophic reference strains. As a result, Green and Bousifield (1982) suggested a distinct taxon, which could be excluded from most of the genera to which they had previously been assigned. The genus *Methylobacterium* was chosen to accommodate this taxon.

2.2 ISOLATION OF METHYLOTROPHS

Methylobacterium species are able to utilize methanol as a sole source of carbon and energy, because of their characteristic pigmentation they can be easily isolated on methanol mineral salts (MMS) as a selective medium (Whittenburry *et al.*, 1970). Since the growth was reported to be more luxuriant, with a deeper pigmentation on glycerol peptone (GP) agar medium was used for subculturing and storing for a longer time at refrigerated conditions (Green, 1992).

2.3 CHARACTERIZATION OF METHYLOTROPHS

2.3.1 Morphological characterization

Patt *et al.* (1976) emended the genus *Methylobacterium*, all strains were rods 0.8 to 1.0 by 1.0 to 8.0 μm, occurring singly or occasionally branched and pleomorphic motile by single polar, subpolar or lateral flagella, although some strains are not vigorously motile. Cells often contain large (poly β-hydroxybutyrate) sudanophilic inclusions and some times volutin granules. Gram negative, although many strains are gram variable, representative strains have the multilayered cell wall structure and the type of citrate-sythase characteristic of gram negative bacteria (Patt *et al.*, 1974).

Colonies on glycerol peptone agar are ≤ 1 to 3 mm in diameter and pale pink to bright orange red, colonies on methanol-salts agar are a uniform pale pink. The pigment is insoluble and probably

carotenoid. The static liquid media strains grow as a pink surface ring or pellicle (Downs *et al.*, 1974).

2.3.2 Biochemical characterization

All *Methylobacterium* strains are catalase and oxidase poisitve, chemoorganotrophss, facultative methylotrophs capable of growing on a variety of C_1 compounds. The methyl red and voges proskauer tests are negative some strains reduce nitrate to nitrite, urease produced by all strains, lipolytic activity of some strains is week. β-galactosidase, L-orinithine, decarboxylase, L-lysine decarboxylase and L-arginine dihydrolase enzymes are not produced (Green, 1992). Facultative methylotrophs are aerobic in nature, possess catalase, oxidase and urease enzymes. The methyl red and voges proskauer test was found to be positive for only two PPFM strains PPFM-RL-3 and PPFM-RL_10. None of the isolates produced carbinol, indole or H_2S or hydrolyzed casein, starch, gelatin or cellulose and few isolates exhibited denitrification potential (Madhaiyan, 2002 and Anu Rajan, 2003).

2.3.3 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 Bousifield (1982) reported 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, 1982).

Green and Bousifield (1982) listed some of the compounds were used by most strains of *Methylobacterium* as carbon and energy sources *viz.*, glycerol, malonate, succinate, fumarate, α-ketoglutarate, DL-lactate, DL-malate, acetate, pyruvate, propylene glycol, ethanol, methanol and formate.

Urakami and Komagata (1984) reported that some strains can also utilize L-arabinose, D-xylose, D-fucose, D-glucose, D-galactose, D-fructose, L-Aspartate, L-glutamate, adipate, Sebacate, D-tartarate, citrate, saccharte, mono-methylamine, trimethylamine, trimethylamine N-oxide, ethanolamine, butylamine, dimethylglycine and betaine, ammonia, nitrate and urea were shown to be nitrogen sources.

2.4 PHYTOHORMONES PRODUCTION IN METHYLOTROPHS

Growth regulators play a crucial role in enhancing the vigour and increasing the productivity of crop plants. Recently, an alternative to the plant growth regulators of inorganic sources, researchers have now focused on microbial source of growth regulators in the context of

sustainability and cost of chemicals (Thangamani and Sundaram, 2005b). 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.

2.4.1 Auxins

Auxins are a group of plant hormones, indole derivatives, which are produced in the apical meristem of plants. One of the most important auxin is indole-3-acetic acid (IAA), which is synthesized from tryptophan. In addition to IAA, plants usually contain other indole-compounds, which are either intermediates of the synthesis of IAA or products of its conversion. These are indole-3-pyruvic acid (IPA), indole-3-lactic acid (ILA), indole-3-acetonitrile, indole-3-acetaldehyde, tryptamine and tryptophol. The auxin activity of indole compounds is due to their conversion to IAA or to their inherent activity.

Like other plant growth regulators, IAA results in diverse physiological effects in plants. It stimulates the division, extension and differentiation of plant cells, enhances root formation by promoting the conversion of parenchyma into xylem and phloem and regulate the leaf fall and fruit ripening (Muromtsev *et al.*, 1987). Many epiphytic and soil bacteria are able to synthesize auxins, primarily IAA

due to which they influenced the growth of plants either beneficially or adversely (Fett *et al.*, 1987) obligately and facultatively methylotrophic bacteria with different pathways of C_1 metabolism were found to be able to produce auxins, particularly indole-3-acetic acid (IAA), in amounts 3-100 μg ml^{-1}. The production of auxins by methylobacteria was stimulated by the addition of L-tryptophan to the growth medium and was inhibited by ammonium ions. IAA presumably synthesized by methylobacteria through indole-3-pyruvic acid (Ivanova *et al.*, 2001).

Madhaiyan (2002) detected IAA from culture filtrate of FM strains by HPLC method and observed few unidentified compounds in addition to IAA and also confirmed that the methylotrophic isolates grown in a nitrate containing medium with L-tryptophan synthesize IAA. The amount of IAA synthesized was found to vary with strain, the addition of tryptophan in the growth medium and crop plants. PPFM strains produced more amount of IAA than NPFM strains. Thangamani and Sundaram (2005b) also reported that IAA production by FMs and it ranges from 0.24 μg ml^{-1} of culture filtrate (RSP9) to 7.34 μg ml^{-1} of culture filtrate (RLP_{12}) without tryptophan and the addition of tryptophan to the growth medium at the rate of 0.05 per cent enhanced the synthesis of indole compounds by the *Methylobacterium* sp. and ranges from 2.19 μg ml^{-1} of culture filtrate (RSP9) to 17.59 μg ml^{-1} of culture filtrate (RLP_{12}). Anu Rajan (2003) reported that the amount of IAA produced by PPFM strain ranged from

1.31 μg ml^{-1} in *Methylobacterium* SpC_{16} to 4.88 μg ml^{-1} in *Methylobacterium* sp. SM_3 and in presence of tryptophan it varies from 3.48 μg ml^{-1} in C_{16} to 8.77 μg ml^{-1} in SM_3. In case of NPFM strain the IAA production ranges from 4.61 μg ml^{-1} of culture filtrate in NPFM-SB-4 to 7.83 μg ml^{-1} of culture filtrate in NPFM-SB-3 (Senthilkumar, 2003).

2.4.2 Gibberellins

Gibberellins are a group of plant growth regulators which at by modifying the plant morphology (Atzorn *et al.*, 1988). GA induces the uptake of minerals like K and Ca, increase the chlorophyll content, soluble sugars and protein content of the plants. Besides that it enhance better growth and faster elongation rate in shoot exhibited due to induction of active hairy root zone (Hamida and Elkomy, 1998). Some of the microorganisms that are reported to produce GA are *Rhizobium leguminosarum* b.v. *phaseoli* (Jansen *et al.*, 1992). *Azospirillum brasilense* and *Azospirillum lipoferum* (Piccoli *et al.*, 1996).

Hamida and Elkomy (1998) reported the ability of *Azospirillum* sp. to alleviate the effect of water deficit in cereal seedling under salt and osmotic stress that can be attributed partly to bacteria GA production (Piccoli *et al.*, 1999). Anu Rajan (2003) reported GA production by *Methylobacterium* sp and the amount of GA production were found to vary with strains ranging from 10.9 μg to 106.97 μg ml^{-1} of the culture broth.

2.4.3 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 that at any time since the discovery of cytokinins. It is an error to assume that microbes neither are present nor contribute to the cytokinin profile of root tissues. Holland and Polacco (1994) reported the probability of covert contaminants inhabit putatively axenic whole or tissues on culture represents an unrecognized cytokinin "wild card" that might resolve some cases of cell habituation and generally explain the uneven growth or performance of cultures. When PPFM population

in seeds are reduced, seed germination declines. This effect can 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 restorers normal roots development (Holland, 1997). Cytokinin biosynthesis in plants has been studied for well over 40 years, yet despite intensive research we are still no closer to understanding the molecular steps involved in this pathway. In addition to well established effects in plants, it is clear that cytokinin flux also plays an important role in the interactions of some microorganisms with plants, thus stimulating further questions about the origins of cytokinins and raising the possibility of the involvement of microorganisms in cytokinin biosynthesis and distribution. More recently, with reference into PPFMs and other intricate plant-microbe interactions, the cyotkinin conundrum seems likely to become even more complex (Ashby, 2000).

Presence of PPFMs in adequate quantities in the growing tissues leads to the cytokinin production. Cytokinins are said to act as signal molecules and they 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).

Another interesting effect of PPFMs is one seed germination. The pink pigmented facultatively methylotrophic (PPFM) bacterium influence 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 of 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 Placco, 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 10^5 to 10^9 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 methylotrophic bateria (PPFMs) are normally associated with seeds and leaves of soybean and all other plants (Freyermuth *et al.*, 1996).

2.4.4 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.

2.5 METHYLOTROPHS AND PLANT GROWTH

Holland (1992) reported a method for treating plants increasing productivity of a plant by spraying pink pigmented facultative

methylotrophic (PPFM) bacteria on a plant. The invention also relates to increasing productivity of a plant under stress by applying pink pigmented facultative methylotroph (PPFMs) 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 soybeans. PPFMs 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 in dry weight measurements of the plant two weeks after spraying. Results showed that only in the presence of PPFMs did the application of methanol result in increased growth (Munsanje, 1996). PPFMs could be used as seed inoculums or in seed coatings designed to enhance germinability, storability or vigour of the seeds.

Senthilkumar *et al.* (2002) studied the compatibility of a pink pigmented facultative methylotrophs. The *Methylobacterium* sp. Co47 was found compatible with all microorganisms used as bioinoculants *viz.*, *Rhizobium* sp. COC10, *Azospirillum lipoferum* AZ 204, *Bacillus*

megatherium var *phosphaticum* PSB1, *Psudomonas fluorescens* PF1, *Trichoderma viride* TV6 and *Aspergillus awamori* PSF1 except *Trichoderma harzianum* TH1. All the organisms grew well on glycerol peptone agar, which is also a standard medium for pink pigmented facultative methylotrophs (PPFMs). Similarly the PPFMs also showed moderate growth in media used for growing other bioinoculants. The compatible nature of these organisms established the potential of PPFMs as a new component to prepare mixed bioinoculants for various crops.

Madhaiyan *et al.* (2005a) studied the effect of pink-pigmented facultative methylotrophic 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 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.

2.6 EFFECT OF COMBINED INOCULATION OF BIOINOCULANTS ON PLANT GROWTH AND YIELD OF CROP PLANTS

The combined inoculation or the application of bioinoculants as microbial consortia was found to be better in increasing the growth and yield of crop plants. Paulraj (2002) studied the effect of microbial consortia azophos (constitute 50% of each azospirillum and phosphate solubilizing bacteria) on raising cardamom seedlings under *in vitro* condition and reported that the combined inoculation of *Azospirillum* and phosphate solubilizing bacteria as azophos was found to be better in increasing the germination percentage and vigour index of cardamom than individual inoculation. Thangamani and Sundaram (2005c) have also reported that seedling dip of tomato seedlings in microbial consortia increased the growth and yield parameters of tomato hybrid over individual inoculation and uninoculated control. Ramarethinum and Rajagopal (1998) and Holland (1997) established the cumulative effect of bioinoculants in tea, soybean and coffee respectively. The seed treatment with *Methylobacterium* sp along with other bioinoculants was found to have significant and consistent stimulatory effects on rice seed germination and vigour index (Thangamani and Sundaram, 2005a).

Material and Methods

III. MATERIAL AND METHODS

The present investigation was carried out during the year 2004-06 in the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, on studies of Methylotrophs and their beneficial effects on soybean. The details of the material used and methods followed in the present study are detailed in this chapter.

3.1 COLLECTION OF SAMPLES

Seed samples, soil samples and leaf samples were collected from different crops like *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Cajanus cajan*, *Vigna mungo*, *Vigna radiata*, *Glycine max*, *Helianthus annuus*, *Arachis hypogaea*, *Gossypium hirsutum*, *Lycopersicon esculentum*, *Solanum melongena*, *Abelmoschus esculentus*, *Capsicum annuum*, *Brassica oleraceae* var *capitata*, *Brassica oleraceae* var *botrytis*, *Saccharum officinarum*.

Samples were collected from Dharwad and Belgaum districts. The samples were brought in polythene bags.

3.1.1 Reference culture collection

The reference culture *Methylobacterium* LE-1 was obtained from Dr. S. P. Sundaram, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

3.2 ISOLATION AND PURIFICATION OF METHYLOTROPHS

Methanol mineral salts (MMS) or Ammonium Mineral Salts (AMS) medium (Whittenbury *et al.*, 1970) is a selective medium for

isolation of methylotrophs. 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 1.5-2.0 per cent oxoid purified agar before autoclaving.

3.2.1 Isolation techniques

Leaf imprinting technique (Corpe, 1985)

The upper and lower surface leaf imprints of fresh leaf samples were made on the solidified media separately and incubated at 28±2°C for 5 days.

Serial dilution technique

One gram of sample (soil, seed, leaf) was grinded using pestle and mortar, serially diluted upto 10^{-6} dilutions and one ml of the aliquot from 10^{-4}, 10^{-5} and 10^{-6} dilutions was transferred to the 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^{-1}) 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 methylotrophs were purified by the streak plate method and well isolated colonies on the plates were preserved in MMS 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 METHYLOTROPHS

All the isolates were examined for their cell size, cell shape, motility studies gram reaction and accumulation of PHB.

3.3.1 Cell size

The 72 h grown facultative methylotrophs isolates were observed microscopically using occular and stage micrometer for cell size.

3.3.2 Cell shape (Becking, 1974)

The purified cultures at log phase were observed microscopically for the cell morphological characters.

3.3.3 Motility studies

The 72 h grown FM isolates were observed microscopically using cavity slide for bacterial motility.

3.3.4 Gram reaction (Rangaswami and Bagyaraj, 1993)

Gram staining was carried out as per Rangaswami and Bagyaraj method. Stains and solutions.

3.3.5 Accumulation of PHB (Ostle and Holt, 1982)

Heat fixed smears of 72 h grown bacterial cells were stained with the Nile blue A (1% aqueous solution) at 55°C for 10 min in a coplin-staining jar. After staining, the slides were washed with tap water to remove excess stain and with 8 per cent aqueous acetic acid for 1 min. The stained smear was washed and blot dried with bibulous paper, remoistened with tap water and covered with a No.1 glass cover slip.

The cover slip was necessary, as standard immersion oil would extract some of the fluorescent dye and obscure the field with a general yellow fluorescence. The cover slip thus protects the stained cells from immersion oil. The preparation was examined with a Nikon Labphot microscope with episcopic fluorescence attachment. The Nikon blue excitation method, providing an excitation wave length of approximately 460 mm was used.

3.4 BIOCHEMICAL CHARACTERIZATION

3.4.1 Oxidase test (Cappuccino and Sherman, 1996)

The FM 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 colour change from pink to maroon and finally to purple within 30 sec indicating positive reaction.

3.4.2 Catalase activity (Gerhardt *et al.*, 1981)

A loopful of 24 h old culture of FM 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 and observed for effervescence.

3.4.3 Urease test (Cappuccino and Sherman, 1996)

Urease test was performed on 5 ml of 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 colour indicates a positive reaction for the test.

3.4.4 Casein hydrolysis (Smibert and Kreig, 1981)

The FM isolates were streaked on skim milk agar plates and incubated at room temperature. Hydrolysis of casein was tested and the plates were observed for the presence of clear zones surrounding the colonies and considered as positive reaction.

3.4.5 Starch hydrolysis (Seeley and Vandemark 1981)

The FM isolates were streaked on nutrient agar plates containing 2 per cent insoluble starch and incubated at room temperature. Hydrolysis of starch was tested by flooding with iodine solution and the plates were observed for the presence of clear zones surrounding the colonies and considered as positive reaction.

3.4.6 Cellulose degradation (Seeley and Vandemark, 1981)

The FM isolates were streaked on Czapadox mineral salt agar medium (CMS) containing carboxy methyl cellulose (CMC) and incubated at 35°C in inverted position for 2-5 days. Degradation of cellulose was tested by flooding the plates with 1 per cent aqueous solution of hexadecyl trimethyl ammonium bromide and the plates were observed for the presence of clear zones surrounding the colonies and taken as positive reaction.

3.4.7 Citrate utilization test (Seeley and Vandemark, 1981)

The FM isolates were inoculated into test tubes having Simmons citrate agar medium and incubated for 48 hat 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.

3.4.8 Voges Proskauer test (Seeley and Vandemark, 1981)

The MR-VP broth was inoculated with the FM isolates and incubated for 48 h at 35°C. A quantity of 0.5 ml of α-naphthol solution (5% solution in 70% ethyl alcohol) was added and shaken gently for 15 minutes. The positive reaction of acetyl methyl carbinol production was indicated by development of red colour.

3.4.9 Indole production (Seeley and Vandemark, 1981)

The FM isolates were inoculated into sterilized glucose tryptone broth taken in test tubes. After 48 h of incubation, 0.3 ml of Kovac's

reagent was added and mixed well. The reddening of the alcohol layer within few minutes indicated indole production.

3.4.10 Nitrate reduction test (Cappuccino and Sherman, 1996)

The FM strains were cultivated in 10 ml of nitrate broth at room temperature. After 14 days, 2 ml of the broth was tested by adding equal amounts of sulfanilic acid and alpha naphthylamine. Development of red colour indicated that nitrate had been reduced to nitrite.

3.4.11 Carbon source utilization test (Green and Bousifield, 1982)

AMS liquid medium was prepared with the following carbon compounds *viz.*, glucose, D-galactose, dichloromethane, chloroform and glycerol substituted for methanol at 0.5 per cent level and inoculated with one per cent (v/v) standard inoculum (10^9 cfu ml^{-1}) and incubated in an incubator shaker (150) at 30°C for 5 days and compared to a negative control containing no added carbon source. The long incubation time was needed because of the slow growing nature of cultures on certain carbon compounds.

3.5 SCREENING THE EFFICIENCY OF FM ISOLATES BASED ON THE PRODUCTION OF BENEFICIAL GROWTH PROPERTIES

3.5.1 Indole acetic acid production

The IAA production by FM isolates under *in vitro* condition was determined following the method of Ivanova *et al.* (2001). One hundred

ml quantities of AMS liquid medium were dispensed in 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 mg l^{-1}, one ml of the standard inoculum (10^9 cells ml^{-1}) of FM isolates was added 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.

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

A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Salper's reagent and incubated in darkness for 1 hr at 28°C. The intensity of the pink colour developed was read in 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 μg ml^{-1} of culture filtrate.

3.5.2 Gibberellic acid (GA) production

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

Three to four days old FM culture was centrifuged for 15 min at 10,000 rpm and the supernatant was taken. The cell pellet was re-

extracted with phosphate buffer (pH 8.0) and again centrifuged. Both 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°C and the residue redissolved in 2 ml of distilled water containing 0.05 per cent of Tween 80.

Spectrophotometric 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°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 $\mu g\ ml^{-1}$ broth.

3.5.3 Nitrogen estimation (microkjeldahl method)

To a 250 ml conical flask 100 ml of the N-free malate medium was dispensed and autoclaved. Later one ml of 24 h old culture inoculum was added to each flask. The flasks were incubated at 37°C for seven days.

After 7 days of incubation the culture was homogenized and 10 ml was digested with 5 ml of concentrated H_2SO_4 along with 0.2 g digestion catalyst mixture K_2SO_4 : $CuSO_4$: selenium (100:10:1). After cooling, volume was made upto 10 ml with distilled water. Later ten ml of aliquot was transferred to microkjeldhal distillation unit. The sample was mixed with 20 ml of 40 per cent NaOH and distilled. Ammonia evolved was trapped in four per cent boric acid mixed indicator (Bromocresol green 0.066 g and methyl red 0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against 0.05 N H_2SO_4 and total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

$$\text{Per cent N} = \frac{\text{Titre value x 0.014 x N of } H_2SO_4 \text{ x Vol made}}{\text{Sample used}} \text{ X 100}$$

3.5.4 Cytokinin production

Extraction of cytokinin (Tien *et al.*, 1979)

AMS liquid medium was prepared and inoculated with one per cent (v/v) standard inoculum (10^9cfu ml^{-1}) and incubated in an incubator shaker (150 rpm) at 30°C for 7 days. The culture was centrifuged at 1200 rpm and the cell free culture filtrate was extracted twice with equal amount of n-butanol. The supernatants were pooled. The n-butanol fractions were kept for evaporation. After evaporation

the cytokinin fraction was dissolved in 2 ml of HPLC grade methanol and filter sterilized using 2 μm bacterial filter.

Thin layer chromatography of cytokinin

TLC plates were coated with silicagel – G 1 mm thickness using a spreader. After air drying, the plates were activated by exposure at 100°C for 15 min in a hot air oven. A quantity of 200 μl of the crude cytokinin extract was spotted on the plate at equal distance along with the standard zeatin of 20 ppm concentration. The plates were developed in a solvent system of isopropanol : benzene : ammonia (4:1:1) solvent system. Individual spots on the TLC plates were observed under UV exposure and Rf values of the individual spots were calculated. Each spot was collected from the plates and stored in a clean glass vial.

$$\text{Rf value} = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}} \times 100$$

Quantification of cytokinin compounds was done by taking spectrophotometric reading at 269 nm.

3.5.5 Siderophore production (Reeves *et al.*, 1983)

The FM isolates were grown in AMS liquid medium for 7 days in an incubator shaker at 250 rpm at 28°C. The culture was centrifuged at 12000 rpm for 30 min and the 20 ml cell free culture filtrate was extracted twice with equal amount of ethyl acetate after adjusting the

pH to 2.0 with 0.1 N HCl. The top phase was evaporated to dryness and dissolved in 5 ml distilled water. Five ml of hathway reagent (added 1 ml of 0.1 M ferric chloride and 1 ml of 0.1 N HCl to 100 ml of distilled water followed by 1 ml of 0.1 M potassium ferricyanide) was added to the assay solution and allowed to stand for the colour to develop. To estimate catechol type of siderophore, the absorbance was read at 700 nm with 2, 3-dihydroxy benzoic acid as standard.

3.6 POT CULTURE EXPERIMENT

Pot culture experiment was conducted to know the effect of methylotrophos on growth and yield of soybean under green house condition at Department of Agricultural Microbiology, UAS, Dharwad during 2006 as described below.

3.6.1 Preparation of pots

The medium black soil collected from E block of Main Agricultural Research Station, University of Agricultural Sciences, Dharwad was mixed thoroughly, sieved and filled in the earthernpots of 30 cms diameter at the rate of 8 kg per pot. The required quantity of FYM (90 g pot^{-1}) was weighed separately for each pot and incorporated into the soil.

3.6.2 Properties of soil

Some of the physico-chemical properties of soil is presented in Appendix II.

3.6.3 Fertilizer application

The recommended dose of fertilizers for soybean was 40:80:25 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 as basal dose at the time of sowing.

3.6.4 Sterilization and packaging of carrier

One hundred g of lignite powder was neutralized with 5 g of $CaCO_3$ and mixed thoroughly. The properly mixed lignite powder was packed in sterilizable polyprophylene (PP) bags and sterilized in an autocloave at 121°C for one hour.

3.6.5 Inoculation to the carrier

The inoculum of bioinoculants was prepared by inoculating 72 h old log phase cultures of bioinoculants *viz.*, *Bradyrhizobium* (strain SB_{120}) in 100 ml of YEM broth and *Methylobacterium* in 100 ml of AMS broth (Whittenbury *et al.*, 1970). The flasks were kept on shaker at 28±2°C for five days. The culture broth was then mixed with pre-sterilized lignite powder at the rate of 30 ml per 100 g carrier in case of single inoculation, while in case of combined inoculation 15 ml of *Bradyrhizobium* strain SB_{120} and 15 ml of *Methylobacterium* isolates was added per 100 g of carrier. The treatments are as follows along with different combinations of treatments.

T_1 – ML_2

T_2 – ML_{55}

T_3 – ML_{66}

T_4 – Reference strain LE-1

T_5 – *Bradyrhizobium* SB_{120}

T_6 – ML_2 + SB_{120}

T_7 – ML_{55} + SB_{120}

T_8 – ML_{66} + SB_{120}

T_9 – Reference strain LE-1 + SB_{120}

T_{10} – Control (Uninoculated)

3.6.6 Seeds and sowing

Seeds of soybean var. JS-335 obtained from AICRP on soybean, Main Agricultural Research Station, University of Agricultural Sciences, Dharwad.

The following methylotrophic bacterial isolates *viz.*, ML_2 isolated from the phyllosphere of chilli, MR_{55} isolated from the rhizosphere of sugarcane, ML_{66} isolated from phyllosphere of tomato crop.

Before sowing, seeds were treated with *Methylobacterium* strains and/or *Bradyrhizobium* biofertilizers @ 500 g per ha, as per treatment schedule. Five seeds were sown in each pot and after germination thinning was done to maintain three seedlings per pot. Five replications were maintained for each treatment. Optimum soil moisture was maintained during the experimental period by regular

watering. Necessary plant protection measures were taken as per the recommended practices.

First, second and third samplings were done at 30, 45 and 60 days after sowing for growth and nutrient uptake studies. After the harvest, the plants were immediately washed with tap water, followed by 0.1 N HCl and with double distilled water. The plant samples were air dried for two days and then in hot air oven at 65°C till constant weight was obtained. Later the weight of dry matter was recorded. The plant sample was then powdered in an electrical grinder and powdered plant sample was preserved for further elemental analysis.

3.6.7 Phyllosphere spray of *Methylobacterium* isolates

The *Methylobacterium* cultures (1 x 10^9 CFU ml^{-1} of culture) were diluted to 1:100 and sprayed @ 25 ml/plant during flowering stage.

3.6.8 Observations

Without disturbing the root system the soybean plants were pulled out at 30, 45 and 60 DAS and their root and shoot system were separated and subjected for following observations.

Number of leaves

The number of leaves from each plant were counted and expressed as number of leaves per plant.

Shoot and root length

The length of the shoot and root were recorded after separating it and expressed in cm.

Number of nodules per plant

The number of nodules on root of each plant was counted and their mean was expressed as number of nodules per plant at 45 and 60 DAS.

Nodule dry weight

Nodules were separated from the root system and dried to constant weight in hot air oven at 65°C. Dry weight was recorded and expressed as mg per plant (mg/plant).

Shoot and root dry weight

The dry weight of the shoot and root were recorded after separating it and expressed as gram per plant (g/plant).

Number of pods per plant

The number of pods per plant was counted and their mean was expressed as number of pods per plant at 45 and 60 DAS.

Grain yield (g/plant)

Yield of soybean grains per plant was recorded at the time of harvest.

3.7 ESTIMATION OF CHLOROPHYLL

The total chlorophyll content was determined by using dimethyl sulfoxide (DMSO) method given by Shoef and Lium (1976).

Fresh leaf samples (10 mg) were incubated in 7.0 ml of DMSO at 65°C for 50 minues. At the end of the incubation period, decanted the supernatant and discarded the leaf tissue. Made up the volume of supernatant to 10 ml with DMSO. Read the absorbance of extract at 645 and 663 nm using DMSO as blank.

$$\text{Total chlorophyll (mg/g fresh weight)} = 20.0\ (A_{645}) + 8.02\ (A_{663}) \times \frac{V}{100 \times w \times a}$$

Where,

A = Absorbance at specific wave length (645 and 663nm)

V = Final volume of chlorophyll extract (ml)

W = Fresh weight of sample (g)

a = Path length of light (1 cm)

3.7.1 Estimation of carotenoids (Mahadevan *et al.*, 1986)

Two gram of fresh plant material was ground in a mortar with 20 ml of distilled methanol, filtered the extract on a Buchner funnel through Whatsman No. 42 filter paper. The extraction was repeated until the tissue was free from pigments. Filtrates were pooled and partitioned thrice with equal volume of peroxide free ether using a separating funnel (water was added to produce two layers during initial ether extraction). The combined ether layer (which contains carotenoids) was evaporated under reduced pressure at 35°C in a rotary evaporator. The residue was dissolved in minimum quantity of ethanol, 60 per cent of aqueous KOH was added at the rate of 1 ml for

every 10 ml of the ethanol extract to sapoinify (this will remove chlorophyll, lipids and also cleave the esterified carotenoids). The mixture was boiled for 5-10 min. Equal volume of water was added and partitioned twice with ether. The combined ether layers was evaporated and the residue was dissolved in minimum volume of ethanol. The absorbance of the solution was read at 450 nm and the carotenoid content (mg/100 g) in the sample was quantified using a calibration curve prepared against a high purity β-carotene.

3.7.2 Nutrient uptake studies

The oven dried plant samples were ground to fine powder and used for estimation of nitrogen.

Estimation of nitrogen

The total nitrogen content in the plant sample was estimated following the microkjeldahl method as outlined by Jackson (1973). The analysis was done with 500 mg of oven dried finely ground samples which were digested with five ml of concentrated H_2SO_4 in presence of 200 mg catalyst mixture (containing potassium sulphate, copper sulphate and selenium in 100:10:1 ratio). The samples were digested on a microkjeldahl digestion unit till a clear solution was obtained. The digest was cooled and diluted with distilled water. The digested samples were distilled after adding 20 ml of 40 per cent NaOH to make the digest alkaline in a parnar-wayers type

semimicrokjeldahl distillation unit. The evolved ammonia was absorbed in four per cent boric acid solution and titrated against 0.05N H_2SO_4. A standard was run by using 1 mg of nitrogen per five ml solution of ammonium sulphate and the titre values were converted to mg of nitrogen and per cent nitrogen was calculated.

3.8 POPULATION COUNT

Estimation of phyllosphere and rhizosphere *Methylobacterium* population

One gram of leaf sample was ground using pestle and mortar, serially diluted upto 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. The plates were incubated at 28±2°C, three replications were maintained. The characteristic pink colonies growing over the medium were counted and expressed as 10^{-6} cfu g^{-1} fresh weight of leaf tissue. Similarly population count of methylotrophs from rhizosphere soil was taken and expressed as 10^{-3} cfu 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.

Experimental Results

IV. EXPERIMENTAL RESULTS

A total of 40 strains of methylotrophs were isolated which included 27 from Belgaum and 13 from Dharwad district. The results pertaining to the isolation, purification and characterization of methylotrophs, screening of *Methylobacterium* isolates for beneficial characters and also their effects on soybean plant under pot culture conditions are presented in this chapter.

4.1 ISOLATION OF METHYLOTROPHS FROM DIFFERENT CROPS

Pink pigmented facultative methylobacteria (PPFM) and non-pigmented facultative methylobacteria (NPFM) were randomly isolated from seed surface, rhizosphere and phyllophere of different crop plants collected from Belgaum and Dharwad districts. The isolates from seed surface were designated as MS, isolates from rhizosphere were designated as MR and the isolates from phyllosphere were designated as ML.

The isolates were maintained as the culture bank in the Department of Agricultural Microbiology, UAS, Dharwad.

4.2 MORPHOLOGICAL CHARACTERIZATION

The results of the morphological characterization of both NPFM and PPFM isolates are presented in Table 2.

All the isolates observed were gram negative, rod shaped with the dimension of 0.6-1.0 x 1.0-1.5 μm, accumulate PHB, colonies were pink, pale pink or dark pink in colour due to pigmentation.

Table 1. Tentatively identified methylotroph isolates from various crops

Sl. No.	Place	Source	Crop	Isolates obtained
1.	Saundatti	Phyllosphere	Cotton	ML_1
2.	Saundatti	Phyllosphere	Chilli	ML_2
3.	Belavadi	Phyllosphere	Tomato	ML_4
4.	Belavadi	Phyllosphere	Chilli	ML_5
5.	Tadkod	Phyllosphere	Bendi	ML_6
6.	Tadkod	Phyllosphere	Radish	ML_{10}
7.	Saundatti	Rhizosphere	Maize	MR_{11}
8.	Belavadi	Rhizosphere	Sorghum	MR_{12}
9.	Sadalagi	Rhizosphere	Bean	MR_{14}
10.	Sadalagi	Rhizosphere	Bean	MR_{15}
11.	Davalpur	Rhizosphere	Soybean	MR_{16}
12.	Bagewadi	Phylloplane	Soybean	ML_{18}
13.	Davalpur	Seed surface	Sorghum	MS_{21}
14.	Hebbar	Seed surface	Sorghum	MS_{22}
15.	Hebbar	Phyllosphere	Chilli	ML_{29}
16.	Bedkihal	Phyllosphere	Redgram	ML_{30}
17.	Bedkihal	Phyllosphere	Soybean	ML_{31}
18.	Hukkeri	Phyllosphere	Groundnut	ML_{32}
19.	Sutagatti	Phyllosphere	Sunflower	ML_{35}
20.	Huttargi	Phyllosphere	Groundnut	ML_{36}
21.	Huttargi	Phyllosphere	Sugarcane	ML_{37}
22.	Yaragatti	Phyllosphere	Bendi	ML_{38}
23.	Sutagatti	Phyllosphere	Brinjal	ML_{39}
24.	Doddi	Phyllosphere	Tomato	ML_{40}
25.	Doddi	Rhizosphere	Tomato	MR_{46}
26.	Gungaragatti	Rhizosphere	Maize	MR_{47}
27.	Garag	Phyllosphere	Cowpea	ML_{49}
28.	MK Hubli	Phyllosphere	Sugarcane	ML_{50}
29.	MK Hubli	Phyllosphere	Sugarcane	ML_{52}
30.	Krishinagar	Phyllosphere	Bengalgram	ML_{55}
31.	Krishinagar	Phyllosphere	Tomato	ML_{57}
32.	MK Hubli	Rhizosphere	Sugarcane	MR_{60}
33.	Krishinagar	Rhizosphere	Soybean	MR_{61}
34.	Kalagatagi	Rhizosphere	Groundnut	MR_{62}
35.	Kalagatagi	Rhizosphere	Maize	MR_{64}
36.	Mugad	Phyllosphere	Rice	ML_{66}
37.	Govankoppa	Seed surface	Cotton	MS_{69}
38.	Hebballi	Seed surface	Chilli	MS_{72}
39.	Kalagatagi	Seed surface	Sorghum	MS_{77}
40.	Mugad	Phyllosphere	Rice	MS_{78}

Table 2. Morphological characterization of methylotrophs

Sl. No.	Isolates	Cell size (μm)	Cell shape	Gram reaction	Pigmentation	Accumulation of PHB
1.	ML_1	0.6 x 1.0	Rod	Negative	Light pink	Positive
2.	ML_2	0.6 x 1.2	Rod	Negative	Light pink	Positive
3.	ML_4	0.6 x 1.0	Rod	Negative	Dark pink	Positive
4.	ML_5	0.8 x 1.0	Rod	Negative	Dark pink	Positive
5.	ML_6	0.8 x 1.3	Rod	Negative	Dark pink	Positive
6.	ML_{10}	0.8 x 1.2	Rod	Negative	Dark pink	Positive
7.	MR_{11}	0.6 x 1.3	Rod	Negative	Dark pink	Positive
8.	MR_{12}	0.7 x 1.3	Rod	Negative	Dark pink	Positive
9.	MR_{14}	0.7 x 1.0	Rod	Negative	Light pink	Positive
10.	MR_{15}	0.8 x 1.2	Rod	Negative	Dark pink	Positive
11.	MR_{16}	0.8 x 1.2	Rod	Negative	Light pink	Positive
12.	ML_{18}	0.8 x 1.4	Rod	Negative	Light pink	Positive
13.	MS_{21}	0.6 x 1.3	Rod	Negative	Light pink	Positive
14.	MS_{22}	0.6 x 1.4	Rod	Negative	Light pink	Positive
15.	ML_{29}	0.6 x 1.5	Rod	Negative	Light pink	Positive
16.	ML_{30}	0.6 x 1.3	Rod	Negative	Light pink	Positive
17.	ML_{31}	0.6 x 1.2	Rod	Negative	Light pink	Positive
18.	ML_{32}	0.8 x 1.4	Rod	Negative	Dark pink	Positive
19.	ML_{35}	0.8 x 1.0	Rod	Negative	Light pink	Positive
20.	ML_{36}	0.9 x 1.5	Rod	Negative	Light pink	Positive
21.	ML_{37}	0.9 x 1.2	Rod	Negative	Light pink	Positive
22.	ML_{38}	0.9 x 1.3	Rod	Negative	Light pink	Positive
23.	ML_{39}	0.9 x 1.2	Rod	Negative	Light pink	Positive
24.	ML_{40}	0.9 x 1.1	Rod	Negative	Dark pink	Positive
25.	MR_{46}	0.7 x 1.3	Rod	Negative	White	Positive
26.	MR_{47}	0.8 x 1.4	Rod	Negative	White	Positive
27.	ML_{49}	0.8 x 1.2	Rod	Negative	Dark pink	Positive
28.	ML_{50}	0.7 x 1.2	Rod	Negative	Dark pink	Positive
29.	ML_{52}	0.7 x 1.1	Rod	Negative	Dark pink	Positive
30.	ML_{55}	0.8 x 1.0	Rod	Negative	Dark pink	Positive
31.	ML_{57}	0.8 x 1.3	Rod	Negative	Dark pink	Positive
32.	MR_{60}	0.9 x 1.5	Rod	Negative	White	Positive
33.	MR_{61}	0.9 x 1.2	Rod	Negative	White	Positive
34.	MR_{62}	0.9 x 1.5	Rod	Negative	White	Positive
35.	MR_{64}	0.6 x 1.3	Rod	Negative	White	Positive
36.	ML_{66}	0.6 x 1.4	Rod	Negative	Dark pink	Positive
37.	MS_{69}	0.6 x 1.3	Rod	Negative	Light pink	Positive
38.	MS_{72}	0.8 x 1.4	Rod	Negative	Light pink	Positive
39.	MS_{77}	1.0 x 1.3	Rod	Negative	Dark pink	Positive
40.	MS_{78}	1.0 x 1.2	Rod	Negative	Dark pink	Positive

4.3 BIOCHEMICAL CHARACTERIZATION OF METHYLOTROPH ISOLATES

All the isolates were tested for a selective biochemical tests. All the isolates showed positive results for oxidase test, urease test, indole production. None of the isolates showed positive results for casein hydrolysis, H_2S production, MR and VP test, nitrate reduction test.

4.3.1 Carbon source utilization test

Methylotrophs are able to grow on a wide variety of multi carbon compounds other than single carbon compounds. They were tested for utilization of different carbon compounds. All the isolates showed positive results for utilization of glucose, D-galactose, dichloromethane, chloroform and glycerol.

4.4 SCREENING OF *METHYLOBACTERIUM* ISOLATES FOR GROWTH PROMOTING SUBSTANCES AND OTHER BENEFICIAL CHARACTERS

Methylobacterium isolates were screened based on the production of higher quantity of indole acetic acid, gibberellic acid cytokinin production, nitrogen fixation, P solubilization and siderophore production.

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 is varied with the isolates, results are presented in Table 4.

Table 3a. Biochemical characterization of methylotrophs

Isolates	Oxidase test	Urease test	Nitrate reduction test	MR and VP test	Casein hydrolysis	Starch hydrolysis	Cellulose degradation	Indole production	Citrate utilization	Catalase activity
1 ML_1	+	+	-	-	-	-	-	+	+	+
2 ML_2	+	+	-	-	-	-	-	+	+	+
3 ML_4	+	+	-	-	-	-	-	+	+	+
4 ML_5	+	+	-	-	-	-	-	+	+	+
5 ML_6	+	+	-	-	-	-	-	+	+	+
6 ML_{10}	+	+	-	-	-	+	-	+	+	+
7 MR_{11}	+	+	-	-	-	-	-	+	+	+
8 MR_{12}	+	+	-	-	-	-	-	+	+	+
9 MR_{14}	+	+	-	-	-	+	-	+	+	+
10 MR_{15}	+	+	-	-	-	-	-	+	+	+
11 MR_{16}	+	+	-	-	-	-	+	+	+	+
12 ML_{18}	+	+	-	-	-	-	-	+	+	+
13 MS_{21}	+	+	-	-	-	-	-	+	+	+
14 MS_{22}	+	+	-	-	-	-	-	+	+	+
15 ML_{29}	+	+	-	-	-	+	-	+	+	+
16 ML_{30}	+	+	-	-	-	-	-	+	+	+
17 ML_{31}	+	+	-	-	-	-	-	+	+	+
18 ML_{32}	+	+	-	-	-	-	-	+	+	+
19 ML_{35}	+	+	-	-	-	-	-	+	+	+
20 ML_{36}	+	+	-	-	-	-	-	+	+	+
21 ML_{37}	+	+	-	-	-	-	-	+	+	+
22 ML_{38}	+	+	-	-	-	-	-	+	+	+
23 ML_{39}	+	+	-	-	-	-	-	+	+	+
24 ML_{40}	+	+	-	-	-	-	-	+	+	+
25 MR_{46}	+	+	-	-	-	-	-	+	+	+
26 MR_{47}	+	+	-	-	-	-	-	+	+	+
27 ML_{49}	+	+	-	-	-	+	-	+	+	+
28 ML_{50}	+	+	-	-	-	-	-	+	+	+
29 ML_{52}	+	+	-	-	-	+	-	+	+	+
30 ML_{55}	+	+	-	-	-	-	+	+	+	+
31 ML_{57}	+	+	-	-	-	-	-	+	+	+
32 MR_{60}	+	+	-	-	-	-	-	+	+	+
33 MR_{61}	+	+	-	-	-	-	-	+	+	+
34 MR_{62}	+	+	-	-	-	-	-	+	+	+
35 MR_{64}	+	+	-	-	-	-	-	+	+	+
36 ML_{66}	+	+	-	-	-	+	-	+	+	+
37 MS_{69}	+	+	-	-	-	+	-	+	+	+
38 MS_{72}	+	+	-	-	-	-	-	+	+	+
39 MS_{77}	+	+	-	-	-	-	-	+	+	+
40 MS_{78}	+	+	-	-	-	-	-	+	+	+

Table 3b. Ability of isolates to use different carbon source

Sl. No.	Isolates	Glucose	D-galactose	Dichloro methane	Chloroform	Glycerol
1.	ML_1	+	+	+	+	+
2.	ML_2	+	+	+	+	+
3.	ML_4	+	+	+	+	+
4.	ML_5	+	+	+	+	+
5.	ML_6	+	+	+	+	+
6.	ML_{10}	+	+	+	+	+
7.	MR_{11}	+	+	+	+	+
8.	MR_{12}	+	+	+	-	+
9.	MR_{14}	+	+	+	-	+
10.	MR_{15}	+	+	+	+	-
11.	MR_{16}	+	+	+	+	+
12.	ML_{18}	+	+	+	-	+
13.	MS_{21}	+	+	+	-	+
14.	MS_{22}	+	+	+	+	-
15.	ML_{29}	+	+	+	+	-
16.	ML_{30}	+	+	+	+	+
17.	ML_{31}	+	+	+	+	+
18.	ML_{32}	+	+	+	-	+
19.	ML_{35}	+	+	+	+	+
20.	ML_{36}	+	+	+	+	-
21.	ML_{37}	+	+	+	-	+
22.	ML_{38}	+	+	+	+	+
23.	ML_{39}	+	+	+	+	+
24.	ML_{40}	+	+	+	+	+
25.	MR_{46}	+	+	+	+	+
26.	MR_{47}	+	+	+	+	+
27.	ML_{49}	+	+	+	+	+
28.	ML_{50}	+	+	+	+	-
29.	ML_{52}	+	+	+	+	+
30.	ML_{55}	+	+	+	+	-
31.	ML_{57}	+	+	+	+	+
32.	MR_{60}	+	+	+	+	+
33.	MR_{61}	+	+	+	-	+
34.	MR_{62}	+	+	+	-	+
35.	MR_{64}	+	+	+	+	-
36.	ML_{66}	+	+	+	-	+
37.	MS_{69}	+	+	+	+	+
38.	MS_{72}	+	+	+	+	+
39.	MS_{77}	+	+	+	+	-
40.	MS_{78}	+	+	+	+	-

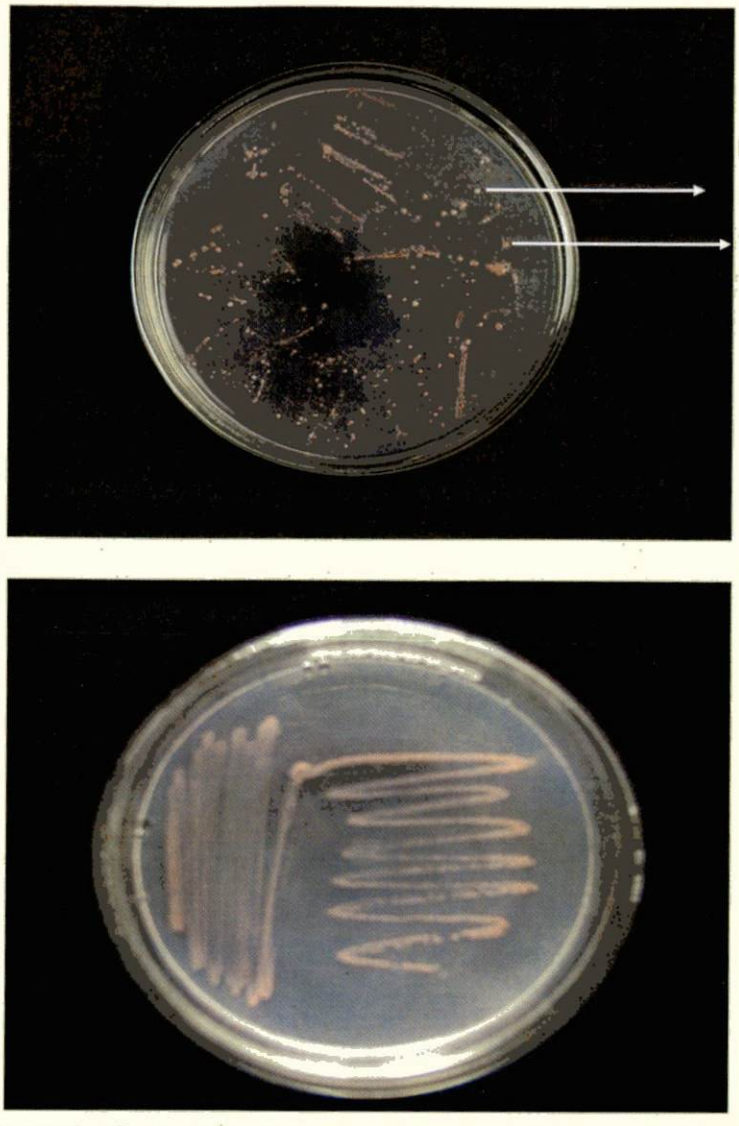

Plate 1. Growth of pink pigmented facultative methylotrophs on AMS medium

Plate 2. Growth of pink pigmented facultative methylotrophs on AMS broth

Table 4. Production of IAA by *Methylobacterium* isolates

Sl. No.	Isolates	IAA (µg/ml)	Sl. No.	Isolates	IAA (µg/ml)
1	ML_1	24.16	21	ML_{37}	14.89
2	ML_2	25.99	22	ML_{38}	23.47
3	ML_4	20.40	23	ML_{39}	10.93
4	ML_5	18.34	24	ML_{40}	17.61
5	ML_6	14.41	25	MR_{46}	15.17
6	ML_{10}	18.30	26	MR_{47}	18.65
7	MR_{11}	18.45	27	ML_{49}	11.28
8	MR_{12}	17.36	28	ML_{50}	13.65
9	MR_{14}	18.92	29	ML_{52}	9.15
10	MR_{15}	15.28	30	ML_{55}	28.15
11	MR_{16}	19.32	31	ML_{57}	18.25
12	ML_{18}	16.49	32	MR_{60}	9.04
13	MS_{21}	21.24	33	MR_{61}	15.85
14	MS_{22}	14.22	34	MR_{62}	15.66
15	ML_{29}	17.46	35	MR_{64}	21.40
16	ML_{30}	24.30	36	ML_{66}	27.50
17	ML_{31}	24.44	37	MS_{69}	22.42
18	ML_{32}	20.41	38	MS_{72}	22.42
19	ML_{35}	23.40	39	MS_{77}	16.42
20	ML_{36}	24.93	40	MS_{78}	13.08
	Reference strain LE-1	30.87			
	S.Em±	0.08			
	CD at 1%	0.31			

The highest indole acetic acid production was recorded in ML_{55} (28.15 μg ml^{-1} of culture filtrate) followed by ML_{66} (27.5 μg ml^{-1} of culture filtrate). Standard strain *Methylobacterium* LE-1 was found to produce (30.87 μg ml^{-1} of culture filtrate). While the lowest indole acetic acid production was recorded MR_{60} (9.04 μg ml^{-1} of culture filtrate).

4.4.2 Gibberellic acid production by *Methylobacterium* isolates

All the isolates were showed positive results for gibberellic acid production. The results are presented in Table 5. The maximum GA production of 70.30 μg ml^{-1} of culture filtrate was observed in ML_{55} followed by ML_{66} (69.31 μg ml^{-1} of culture filtrate). The GA production by standard strain *Methylobacterium* LE-1 was recorded to be 57.24 μg ml^{-1} of culture filtrate. The lowest GA production was observed in ML_{57} (24.11 μg ml^{-1} of culture filtrate) respectively.

4.4.3 Cytokinin production my *Methylobacterium* isolates

The cytokinin production by *Methylobacterium* isolates are presented in Table 6. The zeatin, one of the form of cytokinin was produced by all the isolates except ML_5, ML_6, MR_{11}, MR_{16}, ML_{31}, ML_{36}, ML_{37}, ML_{40}, MR_{64} and ML_{69} respectively. The maximum zeatin production was recorded in ML_2 (10.15 ng ml^{-1} of culture filtrate) followed by ML_{66} (8.24 ng ml^{-1} of culture filtrate) respectively.

The standard strain *Methylobacterium* LE-1 was found to produce zeatin of (10.97 ng ml^{-1} of culture filtrate). While the lowest

Table 5. Production of GA by *Methylobacterium* isolates

Sl. No.	Isolates	GA (µg/ml)	Sl. No.	Isolates	GA (µg/ml)
1	ML_1	57.72	21	ML_{37}	57.04
2	ML_2	54.34	22	ML_{38}	29.00
3	ML_4	58.25	23	ML_{39}	41.01
4	ML_5	57.02	24	ML_{40}	48.91
5	ML_6	60.49	25	MR_{46}	27.12
6	ML_{10}	55.04	26	MR_{47}	70.05
7	MR_{11}	60.13	27	ML_{49}	53.95
8	MR_{12}	41.80	28	ML_{50}	54.41
9	MR_{14}	57.77	29	ML_{52}	44.48
10	MR_{15}	52.84	30	ML_{55}	70.30
11	MR_{16}	53.96	31	ML_{57}	24.11
12	ML_{18}	61.46	32	MR_{60}	30.44
13	MS_{21}	54.17	33	MR_{61}	48.36
14	MS_{22}	48.56	34	MR_{62}	54.18
15	ML_{29}	41.96	35	MR_{64}	62.34
16	ML_{30}	43.18	36	ML_{66}	69.31
17	ML_{31}	48.00	37	MS_{69}	41.51
18	ML_{32}	43.65	38	MS_{72}	54.42
19	ML_{35}	58.62	39	MS_{77}	53.62
20	ML_{36}	57.89	40	MS_{78}	35.04
	Reference strain LE-1	57.24			
	S.Em±	0.22			
	CD at 1%	0.84			

Table 6. Cytokinin production by different *Methylobacterium* isolates

Sl. No.	Isolates	Cytokinin (zeatin) (ng/ml of culture filtrate)	Sl. No.	Isolates	Cytokinin (zeatin) (ng/ml of culture filtrate)
1	ML_1	5.23	21	ML_{37}	2.27
2	ML_2	10.15	22	ML_{38}	6.13
3	ML_4	6.15	23	ML_{39}	5.85
4	ML_5	0.00	24	ML_{40}	0.00
5	ML_6	0.00	25	MR_{46}	0.45
6	ML_{10}	1.22	26	MR_{47}	0.82
7	MR_{11}	0.00	27	ML_{49}	0.93
8	MR_{12}	4.35	28	ML_{50}	5.46
9	MR_{14}	2.31	29	ML_{52}	0.48
10	MR_{15}	1.53	30	ML_{55}	0.75
11	MR_{16}	0.00	31	ML_{57}	0.70
12	ML_{18}	0.78	32	MR_{60}	1.85
13	MS_{21}	0.17	33	MR_{61}	4.83
14	MS_{22}	0.24	34	MR_{62}	0.85
15	ML_{29}	2.35	35	MR_{64}	2.55
16	ML_{30}	1.44	36	ML_{66}	8.24
17	ML_{31}	0.00	37	MS_{69}	7.23
18	ML_{32}	0.16	38	MS_{72}	6.85
19	ML_{35}	0.25	39	MS_{77}	5.26
20	ML_{36}	0.00	40	MS_{78}	4.85
	Reference strain LE-1	10.97			
	S.Em±	0.008			
	CD at 1%	0.032			

zeatin production was recorded in ML_{32} (0.16 ng ml^{-1} of culture filtrate).

4.4.4 Nitrogen fixation by *Methylobacterium* isolates

All the isolates were tested for nitrogen fixation the results are presented in Table 7.

The highest nitrogen fixation was observed in ML_2 (1.187 mg N/g of malate medium) followed by ML_{55} (1.183 mg n/g of malate medium), while the standard strain *Methylobacterium* LE-1 was found to fix (0.680 mg N/g of malate medium). The lowest nitrogen fixation was recorded in ML_{18} (0.247 mg N/g of malate medium).

4.4.5 Siderophore production by *Methylobacterium* isolates

The siderophore production by different isolates are presented in Table 8.

The maximum siderophore production was recorded in ML_2 (0.60 μ moles of α-2, 3, dihydroxy benzoic acid) followed by ML_{66} (0.580 μ moles of α-2, 3, dihydroxy benzoic acid). The standard strain *Methylobacterium* LE-1 was recorded 0.60 of α-2, 3, dihydroxy benzoic acid. While the minimum siderophore production was recorded in ML_{37} (0.240 μ moles of α-2, 3, dihydroxy benzoic acid) respectively.

4.4.6 P-solubilization by *Methylobacterium* isolates

All the isolates were tested for 'P' solubilization but none of the isolates were able to soluiblize tricalcium phosphate (data not shown).

Table 7. Nitrogen fixation by different *Methylobacterium* isolates

Sl. No.	Isolates	Nitrogen (mg/g of malate)	Sl. No.	Isolates	Nitrogen (mg/g of malate)
1	ML_1	0.633	21	ML_{37}	1.093
2	ML_2	1.187	22	ML_{38}	0.997
3	ML_4	0.433	23	ML_{39}	0.930
4	ML_5	0.570	24	ML_{40}	1.323
5	ML_6	0.710	25	MR_{46}	0.853
6	ML_{10}	0.490	26	MR_{47}	0.780
7	MR_{11}	0.850	27	ML_{49}	0.717
8	MR_{12}	1.167	28	ML_{50}	0.557
9	MR_{14}	0.450	29	ML_{52}	0.527
10	MR_{15}	0.383	30	ML_{55}	1.183
11	MR_{16}	0.290	31	ML_{57}	0.557
12	ML_{18}	0.247	32	MR_{60}	0.390
13	MS_{21}	0.820	33	MR_{61}	0.930
14	MS_{22}	0.517	34	MR_{62}	1.173
15	ML_{29}	0.560	35	MR_{64}	0.843
16	ML_{30}	0.757	36	ML_{66}	1.180
17	ML_{31}	1.217	37	MS_{69}	0.430
18	ML_{32}	0.653	38	MS_{72}	0.480
19	ML_{35}	1.173	39	MS_{77}	0.630
20	ML_{36}	1.113	40	MS_{78}	0.570
	Reference strain LE-1	0.680			
	S.Em±	0.003			
	CD at 1%	0.012			

Table 8. Siderophore production by different *Methylobacterium* isolates

Sl. No.	Isolates	µ moles of α-2,3, DHBA	Sl. No.	Isolates	µ moles of α-2,3, DHBA
1	ML_1	0.487	21	ML_{37}	0.240
2	ML_2	0.600	22	ML_{38}	0.280
3	ML_4	0.537	23	ML_{39}	0.360
4	ML_5	0.530	24	ML_{40}	0.470
5	ML_6	0.420	25	MR_{46}	0.440
6	ML_{10}	0.520	26	MR_{47}	0.530
7	MR_{11}	0.280	27	ML_{49}	0.487
8	MR_{12}	0.360	28	ML_{50}	0.380
9	MR_{14}	0.480	29	ML_{52}	0.480
10	MR_{15}	0.500	30	ML_{55}	0.500
11	MR_{16}	0.490	31	ML_{57}	0.530
12	ML_{18}	0.260	32	MR_{60}	0.400
13	MS_{21}	0.477	33	MR_{61}	0.350
14	MS_{22}	0.400	34	MR_{62}	0.460
15	ML_{29}	0.410	35	MR_{64}	0.470
16	ML_{30}	0.417	36	ML_{66}	0.580
17	ML_{31}	0.510	37	MS_{69}	0.360
18	ML_{32}	0.560	38	MS_{72}	0.560
19	ML_{35}	0.517	39	MS_{77}	0.520
20	ML_{36}	0.480	40	MS_{78}	0.472
	Reference strain LE-1	0.603			
	S.Em±	0.001			
	CD at 1%	0.004			

4.5 POT CULTURE STUDIES

Seed treatment of efficient *Methylobacterium* isolates and in combination with *Bradyrhizobium japonicum* strain SB_{120} showed significant influence on different plant parameters and yield component at different growth stages.

4.5.1 Shoot length

Significant differences in the shoot length of soybean observed at 30, 60 DAS (days after sowing) due to various inoculation treatment is presented in Table 9 and Fig. 1.

At 30 DAS, among combined inoculation treatments T_9 (reference strain + *B. japonicum* strain SB_{120}) has recorded significantly higher shoot length (12.60 cm) followed by T_7 (ML_{55} + *B. japaonicum* strain SB_{120}) compared to other treatments. The lower shoot length (8.26 cm) was recorded in T_8 (ML_{66} + *B. japonicum* strain SB_{120}).

Among single inoculation treatments T_4 (Reference strain) recorded maximum shoot length of (11.46 cm) and lowest shoot length of (9.76 cm) was recorded in T_5 (*B. japonicum* strain SB_{120}) whereas treatment T_3 (ML_{66}) which was on par with T_4 (Reference strain). Among both single and combined inoculation treatments T_9 has recorded higher shoot length compared to other treatments.

At 60 DAS, among combined inoculation treatments T_9 recorded significantly higher shoot length (30.33 cm) when compared with rest

Table 9. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot length, root length and number of leaves of soybean at two different growth stages

Treatments	Shoot length (cm)		Root length (cm)		Number of leaves per plant	
	30 DAS	60 DAS	30 DAS	60 DAS	30 DAS	60 DAS
T_1 – ML_2	9.83	24.33	10.50	21.66	10.50	43.00
T_2 – ML_{55}	11.00	24.66	11.91	24.66	10.06	33.00
T_3 – ML_{66}	11.16	26.00	10.32	23.33	10.16	42.33
T_4 – Reference strain	11.46	27.66	5.95	22.66	12.43	44.33
T_5 – *Bradyrhizobium* (SB_{120})	9.76	22.66	7.98	25.33	10.66	45.00
T_6 – ML_2 + SB_{120}	10.26	27.00	7.38	27.00	9.66	46.33
T_7 – ML_{55} + SB_{120}	11.63	30.00	18.41	30.33	12.40	56.66
T_8 – ML_{66} + SB_{120}	8.26	24.66	8.24	29.00	12.13	50.33
T_9 – Reference strain + SB_{120}	12.60	30.33	11.66	29.66	14.23	59.00
T_{10} – Control (uninoculated)	8.46	20.33	5.66	21.33	8.23	31.00
S.Em±	0.08	0.22	0.08	0.21	0.07	0.31
CD at 1%	0.35	0.90	0.32	0.85	0.30	1.28

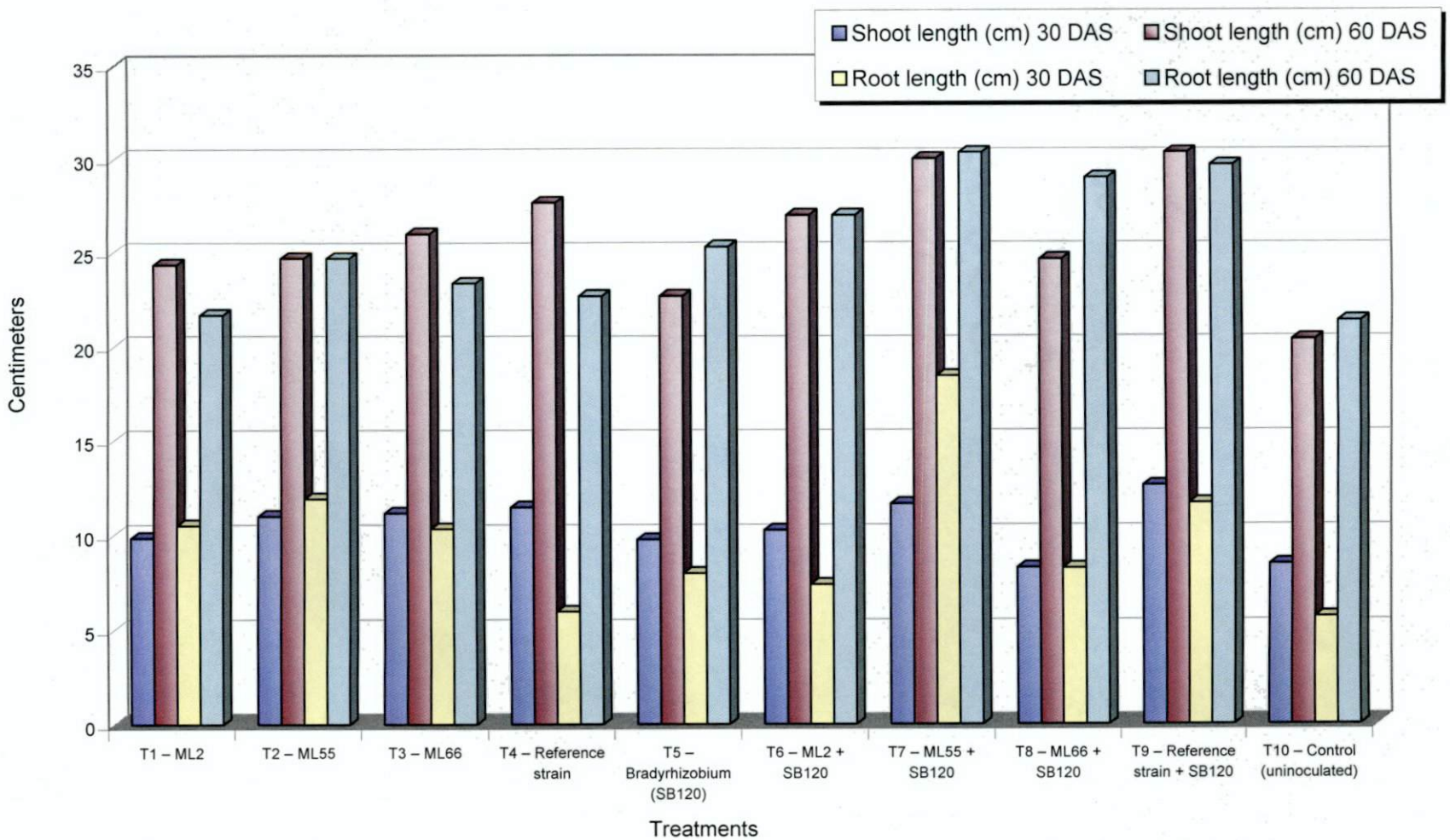

Fig. 1. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot length and root length (cm) of soybean at two different growth stages

of the treatments which was on par with T_7 (30.0 cm) and lower shoot length (24.66 cm) was recorded in T_8 (ML_{66} + *B. japanicum* strain SB_{120}). Among single inoculation treatments T_4 (Reference strain) (27.66 cm) has recorded maximum shoot length and minimum shoot length was with T_5. whereas treatments T_1 and T_2 were on par with each other. Among both single and dual inoculation treatments T_9 has recorded maximum shoot length compared to other treatments and uninoculated control.

4.5.2 Root length

Significant differences of root length was observed due to seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120}. The data pertaining to the root length is presented in Table 9 and Fig. 1.

At 30 DAS, T_7 (ML_{55} + *B. japonicum* strain SB_{120}) recorded significantly higher root length (18.41 cm) followed by T_9 which was recorded (11.66 cm) due to combined inoculation treatments and lower root length (7.38 cm) was recorded in T_6.

Among single inoculation treatments T_2 (11.91 cm) has recorded higher root length followed by T_1 (10.50 cm) and lower root length was recorded in T_4 which was on par with the uninoculated control. Whereas treatments T_1 and T_3 were on par with each other at 30 DAS. Among both single and combined inoculation treatments T_7 has recorded maximum root length.

At 60 DAS, among combined inoculation treatments the maximum root length (30.33 cm) was recorded in T_7 followed by T_9 (29.66 cm) which was on par with the treatment T_8. The minimum root length (27.00 cm) was recorded in T_6. Among single inoculation treatments the higher root length (25.33 cm) was recorded in T_2 followed by T_5 (24.66 cm). The lower root length was recorded in T_1 which was on par with T_4. Among both single and combined inoculation treatments T_7 has recorded maximum root length compared to other treatments and uninoculated control both at 30 and 60 DAS respectively.

4.5.3 Number of leaves

Significant differences in the number of leaves of soybean observed at 30 and 60 DAS due to various inoculation treatments presented in Table 9.

At 30 DAS, among combined inoculation treatment T_9 has recorded significantly higher number of leaves (14.23) followed by T_7 (12.40) which was on par with T_8 (12.13). The lower number of leaves (9.66) was recorded in T_6, whereas among single inoculation treatments T_4 has recorded highest number of leaves (12.43) followed by T_5 (10.66) which was on par with T_3 and T_2 which were 10.16, 10.06, respectively.

At 60 DAS, among the combined inoculation treatments T_9 recorded significantly higher number of leaves (59.00) followed by T_7

(56.66) and the lower number of leaves (46.33) was recorded in T_6. Whereas among single inoculation treatments T_4 has recorded higher number of leaves (45.00) followed by T_1 and lower number of leaves (33.00) was recorded in T_2.

In general, T_9 has recorded highest number of leaves both at 30 and 60 DAS respectively. However uninoculated control showed very low number of leaves at 30 DAS.

4.5.4 Shoot dry weight

Significant variation in the shoot dry weight was observed due to seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120}. The data pertaining to shoot dry weight is presented in Table 10 and Fig. 2.

At 30 DAS, T_9 recorded significantly higher shoot dry weight of 3.76 g per plant followed by T_7 (2.30 g/plant) and lower shoot dry weight was recorded in T_8. Among single inoculation treatments the higher shoot dry weight of 2.51 g per plant was recorded in T_4 followed by T_3 (2.34 g/plant) and the lower shoot dry weight of 1.13 g per plant was recorded in T_1.

At 60 DAS, T_9 recorded significantly higher shoot dry weight of 8.97 g per plant followed by T_7 (8.54 g/plant) and lower shoot dry weight of 6.05 g per plant was recorded in T_8, whereas among single inoculation treatments T_4 has recorded the maximum shoot dry weight of 7.19 g per plant followed by T_3 (7.12 g/plant) and the

Table 10. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot dry weight, root dry weight of soybean at two growth stages

Treatments	Shoot dry weight (g/plant)		Root dry weight (g/plant)	
	30 DAS	60 DAS	30 DAS	60 DAS
T_1 – ML_2	1.13	6.33	0.28	0.48
T_2 – ML_{55}	1.19	6.53	0.24	0.42
T_3 – ML_{66}	2.34	7.12	0.32	0.50
T_4 – Reference strain	2.51	7.19	0.34	0.56
T_5 – *Bradyrhizobium* (SB_{120})	1.16	7.10	0.25	0.51
T_6 – ML_2 + SB_{120}	1.64	7.08	0.30	0.59
T_7 – ML_{55} + SB_{120}	2.30	8.54	0.38	0.67
T_8 – ML_{66} + SB_{120}	1.36	6.05	0.19	0.52
T_9 – Reference strain + SB_{120}	3.76	8.97	0.39	0.68
T_{10} – Control (uninoculated)	0.98	5.89	0.08	0.38
S.Em±	0.01	0.03	0.001	0.001
CD at 1%	0.05	0.13	0.007	0.007

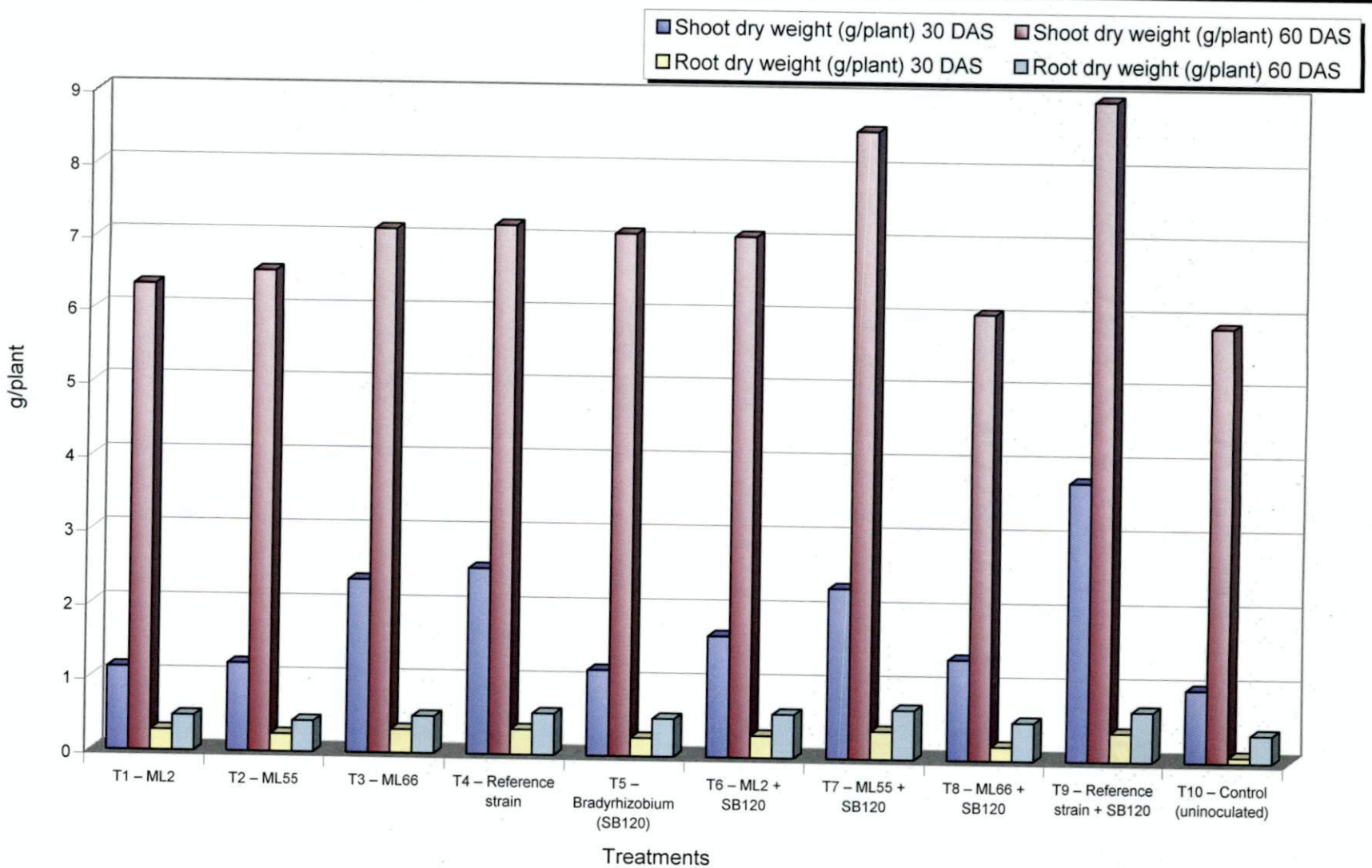

Fig. 2. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot dry weight, root dry weight of soybean at two growth stages

minimum shoot dry weight of 6.33 g per plant was recorded in T_1. Among both single and combined inoculation treatments T_9 has recorded highest shoot dry weight both at 30 and 60 DAS respectively. Whereas uninoculated control recorded lowest shoot dry weight at 30, 60 DAS.

4.5.5 Dry weight of roots

The root dry weight of soybean plants was found to increase significantly due to inoculation of *Methylobacterium* isolates and *B. japonicum* strain SB_{120}. The data regarding the dry weight of roots is presented in Table 10 and Fig. 2.

At 30 DAS, T_9 has recorded the highest root dry weight of 0.39 g per plant followed by T_7 (0.38 g/plant) and the lowest root dry weight of 0.19 g per plant was recorded in T_8 whereas T_9 and T_7 were on par with each other. Among single inoculation treatments significantly highest root dry weight was noticed in the inoculation treatment receiving T_4 (0.34 g/plant) compared with rest of the treatments, however, which was closely on par with T_3 (0.32 g/plant).

At 60 DAS, T_9 showed significantly maximum root dry weight (0.68 g/plant) followed by T_7 (0.67 g/plant) and was significantly superior over rest of the treatments and uninoculated control. The minimum root dry weight was recorded in T_8. Among single inoculation treatments T_4 (0.56 g/plant) recorded highest root dry weight which was significantly superior over rest of the treatments.

The lowest root dry weight was recorded in inoculation treatment receiving T_2. Whereas inoculation treatment receiving T_9 showed significant highest root dry weight when compared to other treatments both at 30 and 60 DAS respectively.

4.5.6 Nodule number

Variations in nodule number at 45 and 60 DAS as consequent of different microbial inoculation and their interaction are presented in Table 11 and Fig. 3.

At 45 DAS, significantly more number of nodules per plant was recorded in T_7 (45.66/plant) followed by T_9 (44.57/plant) when compared with all other treatments and control and significantly lowest nodule number per plant was recorded in control (18.75/plant). Among single inoculation treatments T_2 (39.44/plant) showed significantly superior over rest of the treatments. The lowest number of nodules per plant was recorded in T_3.

Whereas, at 60 DAS, the decreased in nodule number was observed in all the treatments except T_8. The uninoculated control recorded the lowest in nodule number compared to all other treatments. Among single inoculation treatments the more number of nodules per plant was observed in T_5 (39.37/plant) followed by T_3. The less number of nodules per plant was observed in T_1. Both at 45, 60 DAS uninoculated control was inferior over other treatments.

Table 11. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on nodule number and nodule dry weight of soybean at two different growth stages

Treatments	Number of nodules per plant		Nodule dry weight (mg/plant)	
	45 DAS	60 DAS	45 DAS	60 DAS
T_1 – ML_2	29.47	22.34	155.92	152.47
T_2 – ML_{55}	37.45	29.47	198.85	184.44
T_3 – ML_{66}	31.59	35.52	231.91	162.64
T_4 – Reference strain	38.31	28.87	205.81	175.24
T_5 – *Bradyrhizobium* (SB_{120})	39.44	39.37	235.34	194.89
T_6 – ML_2 + SB_{120}	41.54	34.57	195.41	110.62
T_7 – ML_{55} + SB_{120}	45.66	37.52	253.92	216.43
T_8 – ML_{66} + SB_{120}	32.82	33.01	238.71	197.85
T_9 – Reference strain + SB_{120}	44.57	36.50	249.23	210.75
T_{10} – Control (uninoculated)	18.75	17.83	112.67	95.25
S.Em±	0.29	0.16	0.65	0.78
CD at 1%	1.17	0.65	2.62	3.16

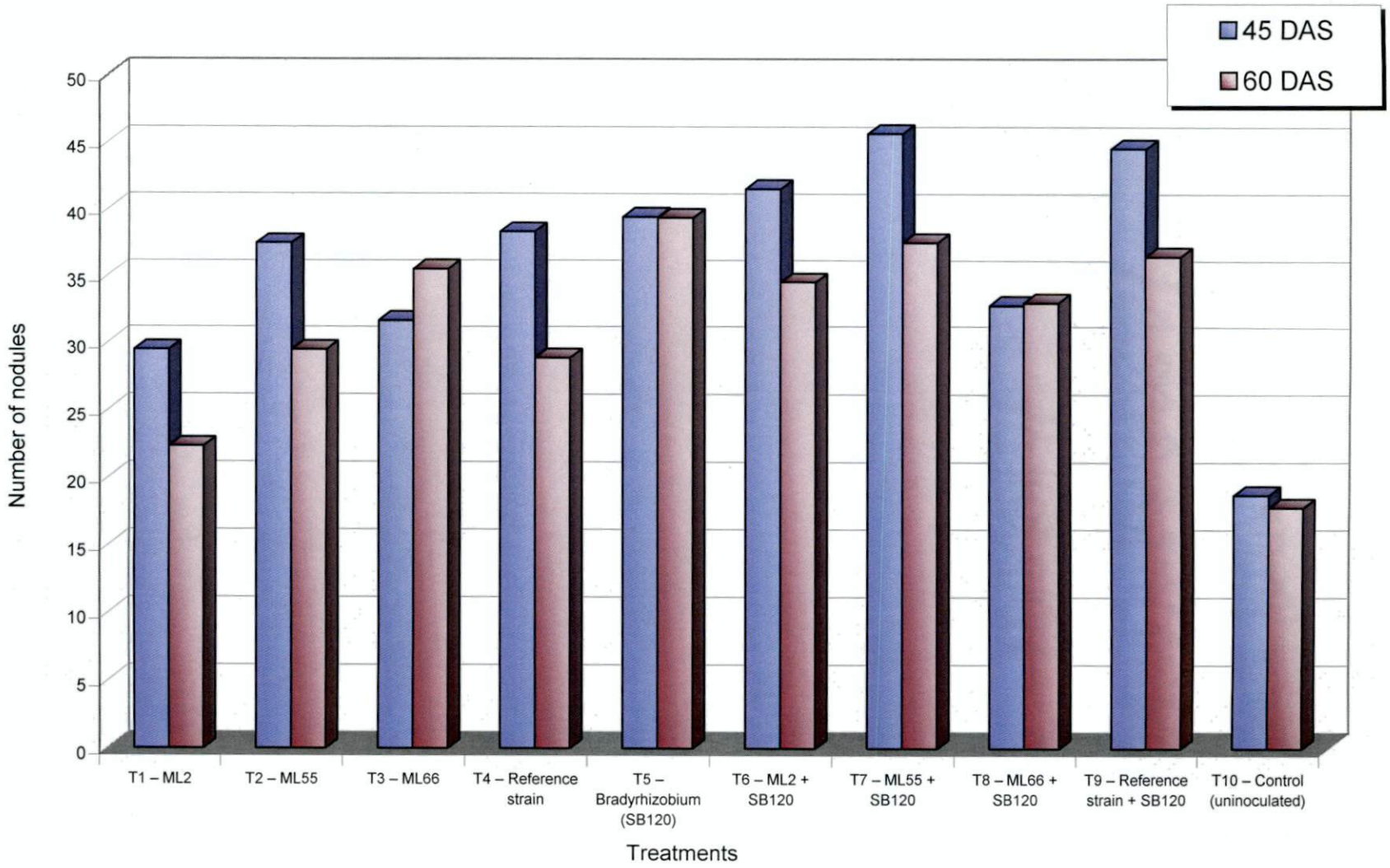

Fig. 3. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on nodule number of soybean at two different growth stages

4.5.7 Nodule dry weight

Significant variation in the nodule dry weight was observed due to seed treatment with *Methylobacterium* isolates and *B. japonicum* strain SB_{120}. The data on the nodule dry weight is presented in Table 11.

At 45 DAS, significantly higher nodule dry weight of 253.2 mg per plant was recorded in T_7 followed by T_9 (44.57 mg/plant). The lower nodule dry weight of 195.41 mg per plant was recorded in T_6. Among single inoculation treatment significantly higher nodule dry weight of 235.34 mg per plant was recorded in T_5 followed by T_2 (231.91 mg/plant) the lowest nodule dry weight of 155.92 mg per plant was recorded in T_1.

At 60 DAS, there was significantly decreased nodule dry weight was recorded in all the treatments. The higher nodule dry weight of 216.43 mg per plant was recorded in T_7 followed by T_9 (210.75 mg/plant) and the lower nodule dry weight of 110.62 mg per plant was recorded in T_6. Among the single inoculation treatments the highest nodule dry weight of 194.89 mg per plant was recorded in T_5 followed by T_2 (184.44 mg/plant). Inoculation treatment receiving T_7 showed significant nodule dry weight compared with uninoculated control both at 45 and 60 DAS respectively.

LEGEND

Plate 3 and 4

T_7 – ML_{55} + SB_{120}

T_9 – Reference strain LE-1 + SB_{120}

T_{10} – Control (Uninoculated)

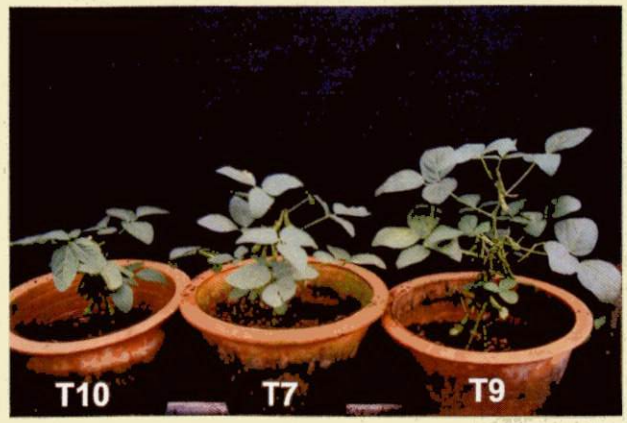

Plate 3. Effect of methylotrophs and *B. japonicum* on plant growth

Plate 4. Effect of methylotrophs and *B. japonicum* on nodulation

4.5.8 Number of pods

Number of pods per plant was significantly increased due to various combined inoculation treatments at 45 DAS and 60 DAS. The data pertaining to the number of pods per plant is presented in Table 12.

At 45 DAS, T_7 showed significantly maximum number of pods per plant (36.24) followed by T_9 (35.71). The minimum number of pods of 30.81 was recorded in T_8. Among single inoculation treatments, T_4 (31.71) recorded more number of pods per plant followed by T_2 (28.13) and was significantly superior over rest of the treatments, whereas T_5 showed less number of pods per plant. Among both single and dual inoculation T_7 has recorded more number of pods per plant. However treatments, T_2 and T_3 were on par with each other at 30 DAS.

At 60 DAS, T_7 (36.90) recorded significantly more number of pods per plant followed by T_9 (36.49) which was on par with T_7. Inoculation treatment receiving T_8 showed less number of pods compared to other treatments. Among single inoculation treatment T_2 (30.84) recorded maximum number of pods per plant followed by T_4 (30.53). Treatment T_1 was on par with the uninoculated control, whereas, inoculation treatment receiving T_5 showed minimum number of pods per plant.

Table 12. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on number of pods and grain yield of soybean

Treatments	Number of pods/plant		Grain yield at harvest (g/plant)
	45 DAS	60 DAS	
T_1 – ML_2	27.39	29.86	10.74
T_2 – ML_{55}	28.13	30.84	11.85
T_3 – ML_{66}	28.07	28.67	10.00
T_4 – Reference strain	30.71	30.53	12.26
T_5 – *Bradyrhizobium* (SB_{120})	27.10	27.32	11.23
T_6 – ML_2 + SB_{120}	31.73	34.47	12.56
T_7 – ML_{55} + SB_{120}	36.24	36.90	15.20
T_8 – ML_{66} + SB_{120}	30.81	33.59	13.53
T_9 – Reference strain + SB_{120}	35.71	36.49	14.72
T_{10} – Control (uninoculated)	26.04	29.25	9.35
S.Em±	0.20	0.23	0.03
CD at 1%	0.81	0.95	0.14

4.5.9 Grain yield

Significant variations in grain yield of soybean plant due to inoculation of different microbial treatment was furnished in Table 12 and Fig. 4.

Significantly highest grain yield was noticed in T_7 (15.20 g/plant) followed by T_9 (14.72 g/plant) which was on par with T_7. Lowest grain yield was recorded in uninoculated control (9.35 g/plant). However, the treatments T_6 and T_8 were on par with each other.

Among single inoculation, higher grain yield was recorded in T_4 (12.26 g/plant) followed by T_2 (11.85 g/plant), whereas treatments T_1 and T_3 were on par with each other. Lower grain yield was recorded in T_3.

4.5.10 Chlorophyll content

Chlorophyll content of soybean was significantly differed at 30 and 60 DAS due to various inoculation treatments and were presented in Table 13.

At 30 DAS, T_9 (2.05 mg/g of tissue) showed significantly maximum chlorophyll content followed by T_7 (1.90 mg/g of tissue) which was on par with T_9. Minimum chlorophyll content (1.06 mg/g of tissue) was recorded in T_6. Among the single inoculation treatments T_2 (1.62 mg/g of tissue) showed more chlorophyll content than other treatments and the less chlorophyll content (0.84 mg/g of tissue) was observed in T_5.

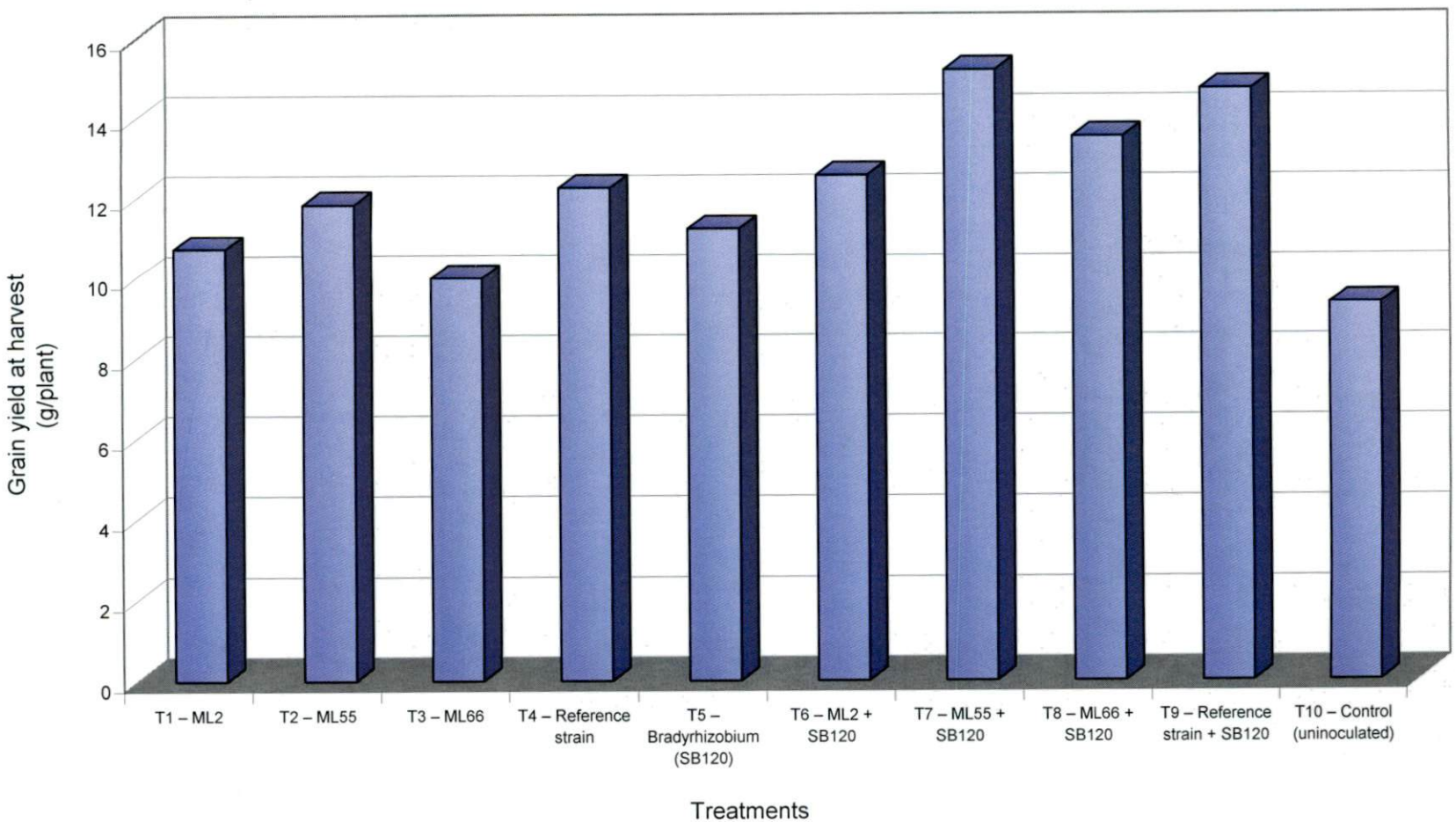

Fig. 4. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on grain yield of soybean

Table 13. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on chlorophyll content and carotenoid content of soybean at two different growth stages

Treatments	Chlorophyll content (mg/g of tissue)		Carotenoid content (mg/g of tissue)	
	30 DAS	60 DAS	30 DAS	60 DAS
T_1 – ML_2	0.96	2.01	0.46	1.23
T_2 – ML_{55}	1.62	2.58	0.47	1.35
T_3 – ML_{66}	1.29	2.51	0.24	1.07
T_4 – Reference strain	1.51	2.57	0.36	1.24
T_5 – *Bradyrhizobium* (SB_{120})	0.84	2.06	0.39	1.12
T_6 – ML_2 + SB_{120}	1.06	2.17	0.46	1.23
T_7 – ML_{55} + SB_{120}	1.90	2.23	0.53	1.46
T_8 – ML_{66} + SB_{120}	1.28	2.60	0.31	1.13
T_9 – Reference strain + SB_{120}	2.05	2.70	0.50	1.40
T_{10} – Control (uninoculated)	0.53	1.73	0.21	0.97
S.Em±	0.006	0.01	0.001	0.006
CD at 1%	0.024	0.06	0.006	0.026

At 60 DAS, significant increase in chlorophyll content was recorded in T_9 (2.70 mg/g of tissue) followed by T_7 (2.60 mg/g of tissue), whereas, among single inoculation treatments, the maximum chlorophyll content was recorded in T_2 (2.58 mg/g of tissue) followed by T_4 (2.57 mg/g of tissue) which was on par with T_4 (2.57 mg/g of tissue).

4.5.12 Carotenoid content

Carotenoid content of soybean was significantly differed at 30 and 60 DAS due to various inoculation treatments and were presented in Table 13.

At 30 DAS, T_7 (0.53 mg/g of tissue) showed significantly maximum carotenoid content followed by T_9 (0.50 mg/g of tissue). The minimum carotenoid content (0.31 mg/g of tissue) was observed in T_8. whereas among single inoculation treatments T_2 (0.47 mg/g of tissue) showed maximum carotenoid content followed by T_4. At 60 DAS, T_7 (1.46 mg/g of tissue) showed significantly maximum carotenoid content followed by T_9 (1.40 mg/g of tissue). The minimum carotenoid content (1.13 mg/g of tissue) was recorded in T_8. Among single inoculation treatment the maximum carotenoid content was recorded in T_2 (1.35 mg/g of tissue) followed by T_4 (1.24 mg/g of tissue) which was on par with T_1. However, uninoculated control recorded minimum carotenoid content compared to other treatment both at 30 and 60 DAS respectively.

4.5.12 Shoot nitrogen content

Significant differences in the shoot nitrogen content of soybean observed at 30, 60 DAS due to various inoculation treatments presented in Table 14 and Fig. 5.

At 30 DAS, T_9 (1.91%) has recorded significantly higher shoot nitrogen content followed by T_7 (1.86%), which was on par with T_8. Whereas lower shoot nitrogen content (1.75%) was recorded in T_6. Among single inoculation treatments, T_5 (1.89%) recorded maximum shoot nitrogen content followed by T_2 (1.82%) which was on par with T_3, whereas minimum shoot nitrogen content (1.45%) was recorded in T_4 (1.45%) which was on par with uninoculated control (1.42%). At 60 DAS, T_9 (2.75%) has recorded significantly higher shoot nitrogen content followed by T_7, whereas the minimum shoot nitrogen content of (1.83%) was recorded in T_6. Among the single inoculation treatments, T_5 (2.63%) has recorded maximum shoot nitrogen content followed by T_2, whereas the minimum shoot nitrogen content of (1.74%) was recorded in T_4 which was on par with the uninoculated control. Treatments T_1 and T_3 were on par with each other at 60 DAS. However, T_4 was on par with the uninoculated control.

4.5.13 Root nitrogen content

Significant differences in the root nitrogen content of soybean was observed at 30, 60 DAS due to various inoculation treatments.

Table 14. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot nitrogen content and root nitrogen content at two different growth stages

Treatments	Shoot nitrogen content (%)		Root nitrogen content (%)	
	30 DAS	60 DAS	30 DAS	60 DAS
T_1 – ML_2	1.64	2.35	0.61	1.48
T_2 – ML_{55}	1.82	2.46	0.80	1.61
T_3 – ML_{66}	1.82	2.26	0.69	1.50
T_4 – Reference strain	1.45	1.74	0.72	1.53
T_5 – *Bradyrhizobium* (SB_{120})	1.89	2.63	0.63	1.49
T_6 – ML_2 + SB_{120}	1.75	1.83	0.74	1.49
T_7 – ML_{55} + SB_{120}	1.86	2.31	0.88	1.65
T_8 – ML_{66} + SB_{120}	1.85	2.16	0.78	1.58
T_9 – Reference strain + SB_{120}	1.91	2.75	0.86	1.63
T_{10} – Control (uninoculated)	1.42	1.74	0.46	1.33
S.Em±	0.007	0.02	0.005	0.01
CD at 1%	0.038	0.08	0.020	0.04

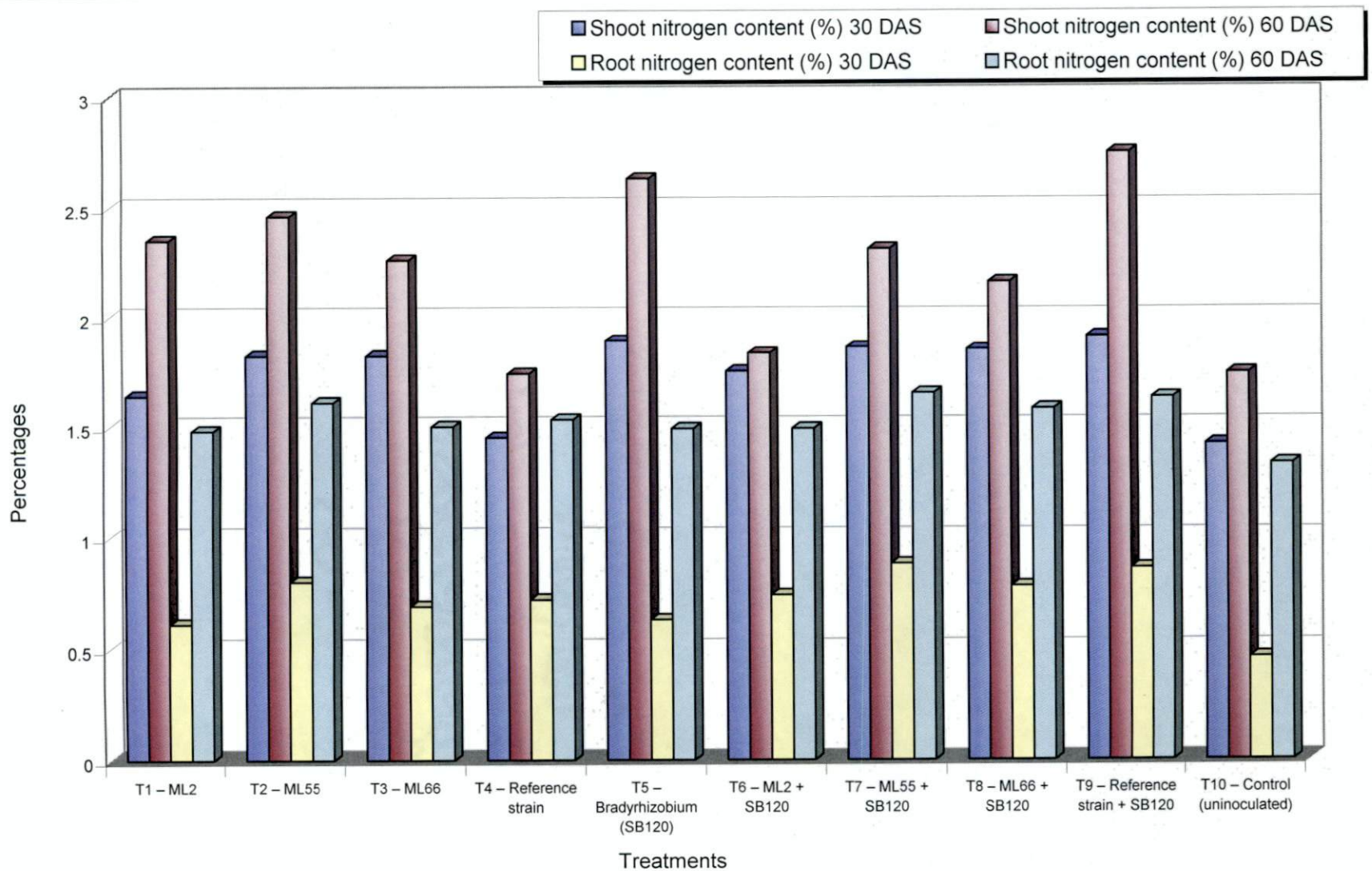

Fig. 5. Influence by seed treatment of *Methylobacterium* isolates and *B. japonicum* strain SB_{120} on shoot nitrogen content and root nitrogen content at two different growth stages

The data pertaining to root nitrogen content is presented in Table 14 and Fig. 5.

At 30 DAS, T_7 (0.88%) has recorded higher root nitrogen content followed by T_9 (0.86%) which was on par with T_7. The lower root nitrogen content (0.74%) was observed in T_6 which was on par with T_8. Whereas, among single inoculation treatments, T_5 has recorded the maximum root nitrogen content (0.80%) followed by T_4. Treatments T_1 and T_2 were on par with each other.

At 60 DAS, the higher root nitrogen content (1.65%) was recorded in T_7 followed by T_9. The lowest root nitrogen content (1.49%) was recorded in T_6. Among single inoculation treatments, T_5 (1.61%) has recorded maximum root nitrogen content followed by T_4 which was on par with T_3 whereas uninoculated control was inferior over other treatments both at 30 and 60 DAS, respectively.

4.6 DYNAMICS OF METHYLOBACTERIUM POPULATION IN PHYLLOSPHERE

The data pertaining to the phyllosphere *Methylobacterium* population is presented in Table 15 and Fig. 6.

The *Methylobacterium* population in phyllosphere was significantly influenced by *Methylobacterium* inoculation.

At 30 DAS, among the dual inoculation treatments, the maximum population of 35.97 x 10^6 cfu g^{-1} fresh weight of leaf tissue

Table 15. Dynamics of population of PPFMs in phyllosphere and rhizosphere of soybean at two different growth stages

Treatments	Phyllosphere (10^6 cfu g^{-1} fresh wt of leaf tissue)		Rhizosphere (10^3 cfu g^{-1} dry weight of soil)	
	30 DAS	60 DAS	30 DAS	60 DAS
T_1 – ML_2	29.10	105.20	15.50	22.17
T_2 – ML_{55}	31.11	110.31	17.97	25.55
T_3 – ML_{66}	30.10	105.64	15.01	24.15
T_4 – Reference strain	30.26	104.34	14.54	23.82
T_5 – *Bradyrhizobium* (SB_{120})	26.84	85.14	12.23	20.43
T_6 – ML_2 + SB_{120}	29.13	95.84	15.08	20.72
T_7 – ML_{55} + SB_{120}	34.89	100.93	16.91	24.24
T_8 – ML_{66} + SB_{120}	33.97	97.66	16.82	23.72
T_9 – Reference strain + SB_{120}	35.97	112.96	18.57	25.31
T_{10} – Control (uninoculated)	17.22	90.81	13.84	19.52
S.Em±	0.21	0.51	0.08	0.17
CD at 1%	0.85	2.07	0.35	0.71

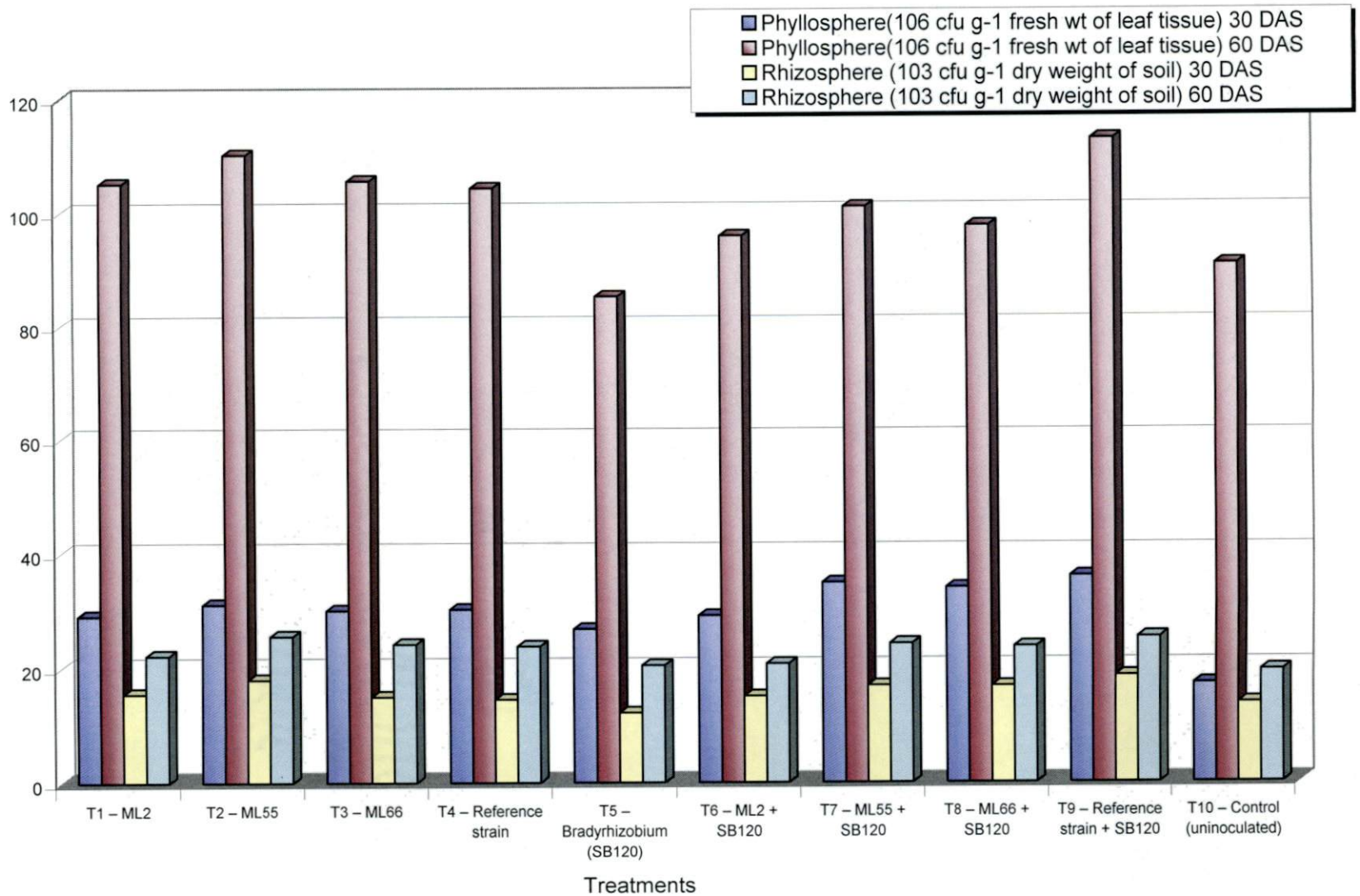

Fig. 6. Dynamics of population of PPFMs in phyllosphere and rhizosphere of soybean at two different growth stages

was recorded in T_9 (Reference strain + SB_{120}) followed by T_7 (ML_{55} + SB_{120}). The minimum population of 29.13 x 10^6 cfu g^{-1} fresh weight of leaf tissue was recorded in T_6, whereas treatment T_9 was on par with T_7.

Among single inoculation treatments T_2 has recorded the maximum population of 31.11 x 10^6 cfu g^{-1} fresh weight of leaf tissue and minimum population of 26.84 x 10^6 cfu g^{-1} fresh weight of leaf tissue was recorded in T_5. However the treatments T_3 and T_4 were on par with each other.

At 60 DAS, there was significant increase in *Methylobacterium* population due to spraying of *Methylobacterium* isolates after 30 DAS, among the dual inoculation treatments the maximum population of 112.96 x 10^6 cfu g^{-1} fresh weight of leaf tissue was recorded in T_9 followed by T_7, when compared with other treatments and uninoculated control. The minimum population was recorded in T_6, whereas among single inoculation treatments T_2 (ML_{55}) has recorded the maximum population of 110.31 x 10^6 cfu g^{-1} fresh weight leaf tissue followed by T_3 (ML_{66}) which was recorded 105.64 x 10^6 cfu g^{-1} fresh weight of leaf tissue. The minimum population of 85.14 x 10^6 cfu g^{-1} fresh weight of leaf tissue was recorded in T_5. However, treatments T_1 and T_3 were on par with each other.

Among both single dual inoculation treatments T_9 has recorded maximum population both at 30 and 60 DAS respectively. Whereas

uninoculated treatment recorded the minimum population compared to other treatments both at 30 and 60 DAS.

4.6.1 Dynamics of *Methylobacterium* population in rhizosphere

The data pertaining to the rhizosphere *Methylobacterium* population is presented in Table 15 and Fig. 5.

At 30 DAS, among the dual inoculation treatments, the maximum rhizoshpere population of 18.57 x 10^3 cfu g^{-1} dry weight of soil was recorded in T_9 followed by T_7 (ML_{55} + SB_{120}). However treatment T_7 and T_8 were on par with each other. The minimum population was recorded in T_6 (ML_2 + SB_{120}).

Whereas among single inoculation treatments, the maximum population of 17.97 x 10^3 cfu g^{-1} dry weight of soil was recorded in T_2 (ML_{55}) followed by T_1 (ML_2) which recorded 15.50 x 10^3 cfu g^{-1} dry weight of soil. However T_1 and T_3 were on par with each other. The minimum population of 12.23 x 10^3 cfu g^{-1} dry weight of soil. However, T_1 and T_3 were on par with each other. The minimum population of 12.23 x 10^3 cfu g^{-1} dry weight of soil was recorded in T_5.

At 60 DAS, among the dual inoculation treatments, the maximum population of 25.31 x 10^3 cfu g^{-1} dry weight of soil was recorded in T9 followed by T_7. The minimum population was recorded in inoculation treatment receiving T_6 (ML_2 + SB_{120}). Whereas among single inoculation treatments, the maximum population of 25.55 x 10^3

cfu g^{-1} dry weight of soil was recorded in T_2 (ML_{55}) followed by T_3 (ML_{66}) which recorded 24.15 x 10^3 cfu g^{-1} dry weight of soil. The minimum population of 20.43 x 10^3 cfu g^{-1} dry weight of soil was recorded in T_5 (SB_{120}) which was on par with T_6. Among both single and dual inoculation treatments T_9 has recorded the maximum rhizosphere population compared to all other treatments and control at 30, 60 DAS, respectively.

Discussion

V. DISCUSSION

The rhizosphere is a complex system consisting of the soil immediately adjacent to the roots, the root surface with its overlying slimecoat and the endorhizosphere. The rhizosphere microbial activity is influenced by root activities such as the exudation of organic substrates like aminoacids, sugars, enzymes carbohydrates, vitamins and several other chemicals. These chemical substances as well as microbial interactions that help in nutrient availability may have a profound influence on component of the soil microflora and thereby could have major effects on the growth and development of plants (Linderman, 1986). Hence, the crop productivity can be improved by manipulating the beneficial rhizosphere microorganisms. To maximize the beneficial plant growth responses, optimal combination of selected microbes is to be used, therefore it is important to identify the effective strains of beneficial microorganisms for the planting situation, based on their compatibility and combined efficacy, both *in vitro* and *in vivo* and employ this consortia of microorganisms in real agricultural situations for efficient management and production to promote plant growth and yield. Contrary to the use of single inoculants, combined inoculation of two or more beneficial and compatible organisms have been shown to work better which indicates that in mixed cultures there is a better interaction of the introduced compatible organisms (Alagawadi and Gaur, 1988; Poi *et al.*, 1989; Patil *et al.*, 1992; Belimov

et al., 1995). Combined inoculations are said to work better than single inoculations based on the principle that greater the diversity and number of inhabitants, the higher the order of interaction and more stable and ecosystem (Higa and Wididana, 1991).

In this context, an attempt was made to know the effect of combined inoculation of *Methylobacterium* isolates and *Bradyrhizobium japonicum* on growth and yield of soybean. The results obtained by this study are discussed in this chapter.

5.1 ISOLATION AND CHARACTERIZATION OF *METHYLOTROPHS* FROM DIFFERENT CROPS

In the present study, 40 methylotrophs were isolated from seed surface, rhizoshpere and phyllosphere of different crops. The morphological characterization revealed that all methylotrophs were rods (0.6-1.0 x 1.0-1.5 μm) gram negative, pigmentation ranging from dark pink to light pink colour.

All *Methylobacterium* strains are rods (0.8-1.0 x 1.0-1.8 μm) which occur singly or occasionally in rosettes. They are often branched, pleomorphic, especially in stationary phase cultures reported by Patt *et al.* (1974).

The 32 PPFMs isolates isolated from different tropical plants, when observed under microscope at 72 h after incubation exhibited rod shaped cell morphology and the older culture (7d) exhibited pleomorphic character which confirmed the above observations. All

strains are motile by a single polar, subpolar or lateral flagellum, although some strains are not vigorously motile, cells often contain large poly β-hydroxy butyrate granules, they are Gram negative, although many strains as gram variable (Green and Bousifield, 1982). Methylotroph isolates were further characterized by a series of biochemical tests, they showed positive results for oxidase test, catalase test, urease test and indole production. None of the isolates showed positive results for casein hydrolysis, H_2S production, MR and VP test, nitrate reduction test. Isolates were also tested for utilization of different carbon sources other than single carbon compounds, they showed positive results for utilization of glucose, D-galactose, dichloromethane, chloroform and glycerol

Similar observations were reported by Thangamani (2005). All the isolates showed positive results for citrate utilization, urease test, catalase activity, oxidase test and indole production. None of the isolates could hydrolyse casein or gelatin, cellulose degradation, lipolytic activity, MR and VP test, nitrate reduction test, denitrification test and H_2S production were negative for all the isolates tested except NPFM-OS-02 and NPFM-OS-04. Starch hydrolysis test was positive for NPFM-OS-03, NPFM-OS-04, NPFM-Co-01 and *Methylobacterium nodulans* ORS-2060. Cellulose degradation was positive for NPFM-OS-05. PPFM are capable of growing of C_1 compounds as well as on a wide range of multicarbon substrates (Green and Bousifield, 1983; Green, 1992).

Anthony (1982) also demonstrated that facultative methylotrophs could utilize different carbon compounds and assimilate C_1 compound via serine pathway. However, when they are grown on complex organic substrates they have complete TCA cycle.

5.2 SCREENING OF METHYLOTROPHS FOR GROWTH PROMOTING SUBSTANCES AND OTHER BENEFICIAL PROPERTIES

5.2.1 Indole acetic acid

In the present study, all the isolates were tested for the production of IAA. The production of IAA is varied with the isolates in the presence of tryptophan which ranges from 9.04 μg ml^{-1} (MR_{60}) of culture filtrate to 28.15 μg ml^{-1} (ML_{55}) of culture filtrate. One of the most important auxins in indole-acetic acid (IAA) which is synthesized from tryptophan. A more detailed analysis showed that Methylobacteria with the serine pathway of C_1 metabolism (*Methylobacterium mesophilicum* and *Aminobacter aminovorans*). The ribulose monophosphate pathway (*Methylobacterium mays*) and the ribulose biphosphate pathway (*Paracoccus kondratievae*) synthesize IAA, ILA and IPA respectively. The serine pathway bacteria, indole-3 acetamide was also detected (Costacurta and Vanderleyden, 1995). The last pathway involves on oxidase (Tryptophan Sidechain oxidase, TSO) oxidizing the side chain of tyrptophan (Oberhansli *et al.*, 1991). IAA is synthesized from tryptophan through tryptamine in three steps

of which only the first step (the decarboxylation of tryptophan) is specific to this biosynthetic pathway. It is surprised that tryptophan decarboxylase and TSO have not yet been found in *Methylobacterium* sp. (Ivanova *et al.*, 2001).

The IAA production was found to be increased in the presence of IAA precursor *viz.*, tryptophan. Ivanova *et al.* (2001), Madhaiyan (2002), Senthilkumar (2003) and Thangamani and Sundaram (2005b) have also reported that the presence of tryptophan would increase the IAA production under *in vitro* conditions.

Many epiphytic and soil microorganisms are able to synthesize and secrete auxin, primarily IAA (fett *et al.*, 1987 and Libbert *et al.*, 1966) due to which they influence the growth of plants either beneficially or adversely (Fett *et al.*, 1987). Such microorganisms like *Azospirillum*, *Rhizobium* and *Pseudomonas* may exert beneficial effects on plants which phytopathogenic *Pseudomonas, Agrobacterium* and *Xanthomonas* bacteria exert adverse effects (Fett *et al.*, 1987). The first report on the production of indole acetic in significant amount by four different methylotrophs was reported by Ivanova *et al.* (2001). The synthesis of indole compounds in methylobacteria was found to be strongly inhibited by ammonium ions, the substitution of KNO_3 for $(NH_4)_2\ SO_4$ in the cultivation medium augmented the amount of synthesized indole by 2 to 15 times, depending on the strain. The addition of tryptophan to the cultivation medium enhanced the

synthesis of indole compound or production of auxin by Methylobacteria (Ivanova *et al.*, 2001).

5.2.2 Giberellic acid

Gibberellic acid is a group of plant growth regulators, which act by modifying the plant morphology (Atzorn *et al.*, 1988). It induces the uptake of minerals like K and Ca, increase the chlorophyll content, soluble sugars and protein content of the plants. Besides that it enhance better growth and faster elongation rate in shoot exhibited due to induction of active hairy root zone (Hamida and Elkomy, 1998). Some of the microorganisms that are reported to produce GA are *Rhizobium leguminosarum* b.v. *phaseoli* (Jansen *et al.*, 1992), *Azospirillum brasilense* and *Azospirillum lipoferum* (Piccoli *et al.*, 1996). Katznelson and Cole (1965) reported the *Pseudomonas fluorescence* produced 1-14 μg ml^{-1} of culture filtrate.

In the present study, all the isolates were found to produce GA, which ranges from 24.11 (ML_{57}) to 70.30 (ML_{55}) μg ml^{-1} of culture filtrate.

Anu Rajan (2003) documented the production of giberellic acid by eight of the PPFM isolates tested and the GA production varies from 10.9 μg ml^{-1} to 106.97 μg ml^{-1}.

5.2.3 Cytokinins

Cytokinins are a class of compounds that defined by their ability to stimulate cell division in plants. Cytokinins are produced by

the microbial symbionts of plants not by plants themselves. Plants have long standing symbiotic relationshisps with specific cytokinin production microbes.

Cytokinin biosynthesis in plants has been studied well for over 40 years, yet despite intensive research we are still no closer to understanding the molecular steps involved in this pathway. In addition to well established effects in plants, it is clear that cytokinin flux also plays an important role in the interactions of some microorganisms with plants, thus stimulating further questions about the origins of cytokinin and raising the possibility of the involvement of microorganisms in cytokinin biosynthesis and distribution. More recently with research in an PPFMs and other intricate plant microbe interactions, the cytokinin conundrum seems likely to became even more complex (Ashby, 2000).

The relationship between plants and associated Methylobacteria is obviously not limited only to commensalisms, since the phytohormones synthesized by plants were found to be necessary for phytopathogenic and phytosymbiotic microorganisms and aerobic Methylobacteria were found to be able to synthesize cytokinin necessary for plants. Presence of PPFM in adequate quantities in the growing tissues leads to the cytokinin production. Cytokinins are said to act as signal molecules and they 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 cytokinin, which was formerly thought to be of plant origin. This theory gains credit due to the fact that cytokinin is not systemic in nature and are only having localized effects (Holland, 1997).

Holland (1997) demonstrated that free living PPFMs produce zeatin and zeatin riboside. Seedlings growing with reduced number of PPFMs have stunted root, inoculation of such plants with PPFMs or application of cytokinins to such plants restores normal root development. In the present study all the isolates were tested for the production of zeatin. All the isolates produced zeatin except ML_5, ML_6, MR_{11}, MR_{16}, ML_{31}, ML_{36}, ML_{40}. The zeatin production was ranged between 0.16 ng ml^{-1} (ML_{32}) of culture filtrate to 10.15 ng ml^{-1} (ML_2) of culture filtrate.

The present results showed conformity with the report of Long (2000) using immunoaffinity and HPLC purification, they have isolated 22 to 111 ng l^{-1} transzeatin from culture filtrate of PPFM isolates (*Arabiodopsis*, barley, maize and soybean) and from that of reference strain *Methylobacterium extoruens* AM_1.

Similarly (Thangamani, 2005) reported that the all isolates tested were found to synthesis cytokinin except rice isolate NPFM-OS-05 and cotton isolate NPFM-Co-02 (Shepelyakovskaya *et al.*, 1999). The zeatin production ranges from 0.147 ng l^{-1} to 11.270 ng l^{-1} of culture filtrate. The *trans* zeatin production varies from 0.123 ng l^{-1} to

72.220 ng l^{-1} of culture filtrate. The *trans* zeatin riboside production was observed only in NPFM-OS-02 @ 0.253 ng l^{-1} of culture filtrate, Madhaiyan (2002) and Senthilkumar (2003) have also documented the similar results and reported the production of cytokinin in soybean and rice.

5.2.4 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.

Earlier *Methylobacterium* belongs to α-protcobacteria and are grouped under *Pseudomonas*. Specific strains of *pseudomonas* produce siderophore which enhanced the plant growth and acted as biocontrol agent (Leong, 1986).

Holland (1997) has examined the role of PPFM in iron nutrition in *Vicia faba*. Under iron-limited conditions, *Methylobacterium mesophilicum* was found to produce siderophores with either methanol or galactose as the carbon source. The role of PPFM-produced cytokinin in the stimulation and translocation of minerals and organic compounds in leaves, as well as the potential of plant leaves and roots to utilize siderophores produced by *Methylobacterium mesophilicum* have also been discussed.

In the present study, results on the production of siderophores revealed that all the *Methylobacterium* isolates produce catechol type of siderophore ranging from 0.24 μ moles of DHBA (ML_{37}) to 0.60 μ moles of DHBA (ML_2).

Similarly, Anu Rajan (2003) and Senthilkumar (2003) observed catechol type siderophore production by facultative methylotrophs. The siderophore production by PPFM strains varied ranging from 0.176 μmoles of DHBA in *Methylobacterium* sp. CA_2 to 0.647 μmoles of DHBA in *Methylobacterium* sp. SM3. The reference strain *Methylobacterium extorquens* AM1 produced 0.432 μ moles of DHBA. The siderophore production by NPFM strains varies ranging from 0.255 μmoles of DHBA in NPFM-SB-3 to 0.606 μmoles of DHBA in NPFM-AA-4.

5.3 EFFECT OF COMBINED INOCULATION OF *METHYLOBACTERIUM* ISOLATES AND *BRADYRHIZOBIUM JAPONICUM* ON GROWTH AND YIELD OF SOYBEAN

Inoculation of *Methylobacterium* isolates in combination with *Bradyrhizobium japonicum* strain SB_{120} had significant influence on different plant growth parameters, nutrient uptake and yield of soybean.

There was significant increase in the plant growth parameters like shoot length, root length, leaf number, shoot dry weight and root dry weight.

Among different treatments T_9 (reference strain + *B. japonicum* strain SB_{120}) recorded the higher shoot length and higher root length was recorded in T_7 (ML_{55} + *B. japonicum* SB_{120}). Similarly higher shoot and root dry weight was recorded in T_9, in all the parameters lower shoot length, root length and dry weight was recorded in uninoculated control. Basile *et al.* (1969) first demonstrated the growth enhancing effects of PPFMs on plants. In a tissue culture system, they demonstrated that PPFMs produce a diffusible substance that stimulates the growth of *Scapania nemerosa*, a liverwort with which the bacterium is regularly associated. Later they identified this bacteria "fertilizer" as vitamin B_{12}. In the present study, the increased plant growth may be due to the increased amounts of cytokinins in plants due to the inoculation of methylotrophs, this results similar with the results of Holland (1997) on soybean and may be due to the production of vitamin B_{12} by PPFMs as described by Basile *et al.* (1969). The role of cytokinin is to influence the pathway of differentiation which results in increase in plant growth and hence all parameters showed increased trends than the uninouclated control. The fact that methylotrophic bacteria promote seed germination and the growth of seedlings suggests that these bacteria may synthesize not only cytokinins but also other phytohormones for instance auxins (Long, 2000). Analysis made by Ivanova *et al.* (2000) with the Salkowski and Van Urk reagent revealed the formation of indoles including IAA, by all the Methylobacteria studied. The analysis with

Salkowski reagent of the culture liquids of methylobacteria grown in a nitrate containing medium with L-tryptophan showed that the amount of IAA synthesized ranged from 100-120 μg ml^{-1}.

In the present study increased in plant growth may be due to the coordination between auxin and cytokinins allow a balance of growth in the shoots and root system, when roots become more extensive by the action of auxins, then the cytokinins of the plant signals the shoot system to form more branches.

5.3.1 Number of nodules and nodule dry weight

Number of nodules and nodule dry weight was maximum in the treatment 9 (Reference strain + SB_{120}) followed by T_7 (ML_{55} + SB_{120}) at 45 and 60 DAS and was significantly superior over the control. The increase in the nodule number and nodule dry weight attributed can be ascribed to presence of Rhizobia in the legume rhizosphere influencing the legume roots to secrete growth promoting substances, which in turn meight have enhanced the growth of *Methylobacterium in situ* and a synergistic effect may have achieved in case of treatment 9 and 7. This results have shown conformity with the results of (Dorosinky and Kayrov, 1975) they worked on chickpea and observed that inoculation of chickpea with *Rhizobium* has been reported to increase the nodulation, nitrogen fixation and yield of crop.

Whereas at 60 DAS, there was gradual decrease in all nodule parameters, the nodule formation reaches its peak during flowering

stage and senescence of nodules occur as the plant matures. It is mainly due to reduced biosynthesis of leghaemoglobin in nodule as plant matures. Similarly, Schiffmann and Label (1973) reported that nodule senescence occurred as the plant matured because of decreased biosynthesis of leghaemoglobin in peanut nodules.

The maximum nodule number of due to inoculation of two or more beneficial organisms over single inoculation and uninoculated control has been reported (Iruthayathas *et al.*, 1983; Rao and Dhir, 1993; Alagawadi and Guar, 1988; Balachander and Nagarajan, 1999; Singh, 1996 and Sivaprasad and Rai, 1991).

5.3.2 Yield parameters

The yield parameters such as number of pods per plant, test weight and grain yield was recorded highest in treatment 7 (ML_{55} + SB_{120}) followed by treatment 9 (Reference strain + SB_{120}) The increase in yield due to single inoculation with PGPR over control have been well reported (Defreitas *et al.*, 1997; Raut and Masood Ali, 1983; Prabhakaran *et al.*, 1999; Mehta and Ram Mohan Rao, 1996). However, the maximum increase in yield parameters due to dual and combined inoculation of PGPRs as documented (Patil *et al.*, 1992; Belimov *et al.*, 1995; Alagwadi and Gaur, 1988 and Pratibha *et al.*, 1995). The increase in the yield due to compatible nature of *Methylobacterium* and *Rhizobium* which was established by Senthilkumar *et al.* (2002). Due to their compatible nature combined

influence on phyllosphere by methylotrophs which are plant growth promoting phyllosphere (PGPP) bacteria and on rhizosphere by *Rhizobium* which is nitrogen fixing bacterium might have resulted in increased plant growth and yield parameters.

The increase in the yield parameters is because of the several factors such as release of growth promoting substances like IAA and GA, proliferation of beneficial organisms in the rhizosphere, control of plant pathogens in addition to nitrogen fixation. The response might be due to probiotics of mixed culture of two or more types of organisms (Kundu and Gaur, 1980). Similarly, Rice *et al.* (1995) reported that the production of the plant growth regulators like auxins, particularly indole-3-acetic acid (IAA) and indole-3-pyruvic acid (Ivanova *et al.*, 2001), zeatin, zeatin riboside and reacted cytokinins by Methylotrophs (Holland and Polacco, 1994) and IAA production and nitrogen fixation by *Rhizobium* has been reported as the factors that enhances plant growth of legumes, the increase in the vegetative growth of the plant attributed to the increase in the yield of a crop.

This results are similar with the various literatures on the beneficial aspects of pink pigmented *Methylobacterium* sp. as a potent biofertilizer for increasing crop production in soybean (Long, 2000), maize, blackgram, groundnut, sugarcane, rice, cotton, sunhemp (Madhaiyan, 2002), tomato (Anu Rajan, 2003 and Thangamani and

Sundaram, 2005c). Similarly Suresh Reddy *et al.* (2002) worked on the effect of combined inoculation of PPFMs and *Rhizobium* on groundnut ucltivar $Co(Gn)_4$ and observed that there was significantly increase in plant growth, biomass production and yield parameters of groundnut.

In present study, there was significant increase in chlorophyll content was observed in T_9 (Reference strain + SB_{120}) and also increase in carotenoid content was observed in inoculation treatment receiving T_7 (ML_{55} + SB_{120}) due to combined inoculation of *Methylobacterium* isolates and *B. japonicum* strain SB_{120}. The results have shown conformity with observations made by Madhaiyan *et al.* (2004) they observed that the *Methylobacterium* inoculation was found to increase the photosynthetic activity by enhancing the number of stomata, chlorophyll concentration and malic acid content of crops. Paulraj (2003) have also documented varied levels of chlorophyll content in cardamom, rubber and coffee respectively due to bioinoculation.

In case of nutrient uptake, inoculation treatment 9 (Reference strain + SB_{120}) recorded significantly higher N uptake when compared with all other treatments. However, the 'N' uptake was lowest in uninoculated control. The increase in N and P uptake due to combined inoculation of two or more organisms has been documented by several workers (Balamurgan and Gunasekharan, 1996; Patil *et al.*, 1992; Devananda, 2000). Similarly Hoflich *et al.* (1995); Biswas *et al.*

(2000), Senthilkumar (2003) and Holland (1997) have well documented the increase in nutrient use efficiency when inoculated with *Rhizobium leguminosarum* by *trifoli* in wheat, corn, radish, mustard, rice and by PPFMs in soybean respectively.

In the present study the phyllosphere spraying of methylotrophs significantly influenced the phyllosphere and rhizosphere, *Methylobacterium* population after 30 DAS. This was mainly due to growth hormone production by *Methylobacterium* sp. especially high cytokinin production in apical plant tissues and rhizosphere soil. The phyllosphere spraying of PPFMs significantly influenced the *Methylobacterium* population in the phyllosphere of rice crop (Holland, 1997). Similarly Madhaiyan *et al.* (2005b) have recorded yield increases in cotton and sugarcane following foliar spray of methanol or PPFMs.

Summary

VI. SUMMARY

An attempt was made to know the effect of methylotrophs, isolated from different locations on the growth and yield of soybean under pot culture conditions.

Both pink pigmented facultative methylotrophs and non-pigmented facultative methylotrophs were isolated from Dharwad and Belgaum districts of Karnataka. Isolates were characterized based on morphological and biochemical characteristics. They were then screened for their beneficial traits such as production of plant growth promoting substances (IAA, GA and cytokinin) siderophore production, N_2 fixation and phosphate solbuilization. Later, pot culture experiment was conducted.

The data on plant height, nodule number, nodule dry weight, shoot and root dry weight, chlorophyll content, carotenoid content, shoot and root nitrogen content and yield of soybean as influenced by *Methylobacterium* isolates and *Bradyrhizobium japonicum* strain SB_{120} application were recorded. All the isolates are capable of producing IAA and GA, whereas zeatin, one of the form of cytokinin was produced by all the isolates but 10 isolates were incapable of producing zeatin.

The amount of nitrogen fixation by the *Methylobacterium* isolates were found to be in lesser amount. None of the isolates were capable of solubilizing of phosphate. Similarly isolates were also tested for siderophore production, isolates showed catechol type of siderophore productions.

All the inoculation treatments showed better plant growth and yield compared to uninoculated control. Among the inoculation treatments, single inoculation performed better than uninoculated control whereas, combined inoculations performed better over single inoculation treatments. The results of our studies indicate that the combined inoculations of methylotrophs with *Rhizobium* exerts more favourable effect on growth and productivity of soybean than single inoculations. Results can be extrapolated to field conditions.

References

VII. REFERENCES

ALAGAWADI, A.R. AND GAUR, A.C., 1988, Association effects of *Rhizobium* and phosphate solubilzing bacteria on the yield and nutrient uptake of chickpea. *Plant and Soil*, **105**: 241-246.

ANONYMOUS, 1999, FAO, *Statistics Bulletin*, p.8.

ANONYMOUS, 2004, *Annual Report*, All Indian Co-ordinated Research Project on Soybean, Bangalore centre, pp.251-263.

ANTHONY, C., 1982, *The Biochemistry of Methylotrophs*. Academic Press, London, U.K.

ANU RAJAN, S., 2003, Studies on the occurrence of pink pigmented facultative methylotrophs on vegetable crops. *M.Sc.(Agri.) Thesis*, Tamil Nadu Agricultural University, Coimbatore.

ASHBY, A.M., 2000, Biotrophy and the cytokinin conundrum. *Physiology of Molecular Plant Pathology*, **57**: 147-158.

ATZORN, R., CROZIER, A., WHEELER, C.T. AND SANDBERG, G., 1988, Production of gibberellins and indole-3-acetic acid by *Rhizobium phaseoli* in relation to nodulation of *Phaseolus vulgaris* roots. *Planta*, **175**: 532-538.

AUSTIN, B. AND GOODFELLOW, M., 1979, *Pseudomonas mesophilica*, a new species of pink bacteria isolated from leaf surfaces. *International Journal of Systematic Bacteriology*, **29**(1): 373-378.

AUSTIN, B., GOODFELLOW, M. AND DICKINSON, C.H., 1978. Numerical taxonomy of phylloplane bacteria isolated from *Lolium perenne*. *Journal of Gen. Microbiology*, **104**: 139-155.

BADR-EL-DIN, S.M.S. AND MOAWAD, H., 1988, Enhancement of nitrogen fixation in lentil, fababean and soybean by dual inoculation with Rhizobia and mycorrhizae. *Plant and Soil*, **108**: 117-124.

BALACHANDER, D. AND NAGARAJAN, P., 1999, Dual inoculation of vesicular arbuscular mycorrhiza and *Rhizobium* in greengram. *Legume Research*, **22**: 177-180.

BALAMURUGAN, S. AND GUNASHEKARAN, S., 1996, Effect of combined inoculation of *Rhizobium* sp. and phosphobacteria at different levels of phosphorus in groundnut. *Madras Agricultural Journal*, **83**: 503-505.

BASILE, D.V., SLADE, L.L. AND CORPE, W.A., 1969, An association between a bacterium and a liverwort, *Scapania nemorosa*: *Bulletin of the Torrey Botanical Club*, **96**: 711–714.

BECKING, J.H., 1974, Family 2, *Azotobacteriaceae* In: *Bergey's Manual of Determinative Bacteriology*, 8 th ed. (eds.) R.E. Buchanan and N.E. Gibbons. The Williams and Wilkins Co., Baltimore, pp. 253-261.

BELIMOV, A.A., KOJEMIAKOV, A.P. AND CHUVRAL IYEVA, C.V., 1995, Interaction between barley and mixed cultures of nitrogen fixing and phosphate solubilizing bacteria. *Plant and Soil*, **173**: 29-37.

BISWAS, J.C., LADHA, J.K., DAZZO, F.B., YANNI, Y.G. AND ROLF,E B.G., 2000, Rhizobial inoculation influences seedling vigour and yield of rice. *Agronomy Journal*, **92**: 880-886.

BOTTON, H., TURCO, L.F. AND KENNEDY, A.C., 1990, Rhizoplane colonization of pea seedlings by *Rhizobium leguminosarum* and deleterious root colonizing *Pseudomonas* sp. and effects on plant growth. *Plant and Soil*, **123**: 121-124.

BOUSIFIELD, L.J. AND GREEN, P.N., 1985, Reclassification of bacteria of the genus *Protomonas* Urakami and Komagata 1984 in the genus *Methylobacterium* (Patt, Cole and Hanson) emend, Green and Bousfield, 1983. *International Journal of Bacteriology*, **35**: 209.

CAPPUCCINO, J.G. AND SHERMAN, N., 1996, In: *Microbiology : A Laboratory Manual.* The Benjamin/Cunnings Publishing Company Inc. (4th Edition), Menlopark, California.

CHANAPRAME, S., TODD, J.J. AND WIDHOLM, J.M., 1996, Prevention of pink pigmented facultative methylotrophic bacteria (*Methylobacterium mesophilicum*) contamination of plant tissue cultures. *Plant Cell Reports*, **16**(1): 222-225.

COLBY, J. AND ZATMAN, L.J., 1973, Trimethylamine metabolism in obligate and facultative methylotrophs. *Biochemical Journal,* **132**: 101-112.

CORPE, W.A. 1985. A method for detecting methylotrophic bacteria on solid surfaces. *Journal of Microbiological Methods,* **3**: 215-221.

CORPE, W.A. AND RHEEM, S., 1989, Ecology of the methylotrophic bacteria on living leaf surfaces. *Microbiological Ecology,* **62**: 243-248.

COSTACURTA, A. AND VANDERLEYDEN, J., 1995, Synthesis of phytohormones by plant associated bacteria. *Critical Review of Microbiology,* **21**: 1-18.

DEFREITAS, J.R., BANERJEE, M.R. AND GERMIDA, J.J., 1997, Phosphate solubilizing rhizobacteria enhance the growth and yield but not P uptake of canola (*Brussica napus* L.). *Biology and Fertility of Soils,* **24**: 358-364.

DEVANANDA, B.J., 2000, Role of plant growth promoting rhizobacteria on growth and yield of pigeonpea (*Cajanus cajan* L.) cultivars. *M.Sc.(Agri.) Thesis,* University of Agricultural Sciences, Dharwad.

DILEEPKUMAR, B.S. AND DUBE, H.C., 1992, Seed bacterization with fluorescent *Pseudomonas* for enhanced plant growth, yield and disease control. *Soil Biology Biochemical,* **24**: 539-542.

DOROSINKY, L.M. AND KAYROV, A.A., 1975, Effect of inoculation on nitrogen fixation by chickpea, its crop and content of protein. *Mikrobiologiya*, **44**: 1103-1106.

DOWNS, J. AND HARRISON, D.E.F., 1974, Studies on the production of pink pigment in *Pseudomonas extroquesns* NCIB9399 growing in continuous culture. *Journal of Applied Bacteriology*, **37**: 65-74.

DUNLEAVY, J.M., 1988, *Curtobacterium plantarum* sp.nov. is ubiquitous in plant leaves and is seed transmitted in soybean and corn. *International Journal of Systemic Bacteriology*, **39**: 240-249.

DUNLEAVY, J.M., 1990, Urease production by *Methyloabacterium mesophilicum*, a seed transmitted bacterium ubiquitous in soybean. *Presented at 3rd Biennial Conference Molecular Cell Biology Soybean*, Ames Iowa, July 23-25.

FETT, W.F., OSMAN, S.E. AND DUNN, M.F., 1987, Auxin production by plant-pathogenic *Pseudomonas* and *Xanthomonas*. *Applied and Environmental Microbiology*, **53**: 1839-1845.

FREYERMUTH, S.K., LONG, R.L.G. AND MATHUR,S., 1996, Metabolic aspect of plant interaction with commensal methylotrophs, In: *Microbial Growth on C1 Compounds*, Eds. Lidstrom, M.E., Tabita, F.R., Kluwer Academic Publisher, The Netherlands, pp.277-284.

GERHARDT, P., MURRY, R.G.E., COSTILOW, R.N., NESTER, E.W., WOOD, W.A., KREIG, N.R. AND PHILLIPS, G.B., 1981, In: *Manual of Methods of General Bacteriology.* American Society for Microbiology, Washing DC.

GOPALAN, C., RAMASHASTRY, B.V. AND BALASUBRAMANIAN, S.C., 1994, Nutritive value of Indian foods. *Indian Council of Medium Research*, Hyderabad, India.

GREEN, P.N. AND BOUSIFIELD, I.J. 1983, Emendation of *Methylobacterium* Patt, Cole and Hanson 1976, *Methylobacterium rhodinum* (Heumann 1962) comb. Nov.corig; *Methylobacterium radiotolerans* (Ito and Iizuka 1971), Comb.nov.corrig., and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb.nov. *International Journal of Systemic Bacteriology,* **33**: 875.

GREEN, P.N. AND BOUSIFIELD, I.J., 1982, A taxonomic study of some Gram- negative facultatively methylotrophic bacteria. *Journal of Gen. Microbiology,* **128:** 623-625.

GREEN, P.N., 1992, The genus *Methylobacterium.* In: *The Prokaryotes*, Eds. A. Baloes, H.G. Truper, M. Dworkin, W. Harder, and K.H. Schleifer. Springer-Verlag, Berlin, pp.2342-2349.

HAMIDA, M.A. AND ELKOMY, H.M., 1998, Effect of salinity, gibberellic acid and *Azospirillum* inoculation on the growth and nitrogen uptake of *Zea mays. Biological Plant,* **40**: 109-120.

HERNANDEZ, L.G. AND HILL, G.S., 1983, Effect of plant population and inoculation on yield and yield components of chickpea (*Cicer arietinum*). *Proceedings Agronomy Society NZ*, **13**: 75-79.

HIGA, T. AND WIDIDANA, G.N., 1991, Changes in the soil microflora induced by effective microorganisms. *Proceedings of the First International Conference of Kyurei Nature Farming*, Ed. Parr, J.F., Hornick, S.B. and Whitman, C.E., Washington, USA, pp.153-162.

HIGA, T., 1991, Effective microorganisms : A biotechnology for mankind. *Proceedings of the First International Conference of Kyurei Nature Farming*, Washington, USA, Eds. Parr, J.F., Hornick, S.B. and Whitman, C.E., pp.8-14.

HIRANO, S.S. AND UPPER, C.D., 1992, Bacterial community dynamics. In : *Microbial Ecology of Leaves*, (eds.) J.H. Andrews and S.S. Hirano. Springer-Verlag, New York, pp. 271-294.

HOFLICH, G., WIEHE, W. AND HECHT-BUCHHOLZ, C., 1995, Rhizosphere colonization of different crops with growth promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbiological Research*, **150**: 139-147.

HOLLAND, M.A. 1997. Occams razor applied to hormonology. Are cytokinins produced by plants? *Plant Physiology*, **115**: 865-868.

HOLLAND, M.A. AND POLACCO, J.C., 1992, Urease-null and hydrogenase-null phenotypes of a phylloplane bacterium reveal altered nickel metabolism in two soybean mutants. *Plant Physiology*, **98**: 942–948.

HOLLAND, M.A. AND POLACCO, J.C., 1994, PPFMs and other covert contaminants: Is there more to plant physiology than just plant?. *Annual Review Plant Physiology and Plant Molecular Biology*, **45**: 197-209.

IRUTHAYATHAS, E.E., GUNASEKARAN, S. AND VLASSAK, K., 1983, Effect of combined inoculation of *Azospirillum* and *Rhizobium* on nodulation and nitrogen fixation of wingedbean and soybean. *Scientia Horticulturae*, **20**: 231-240.

IVANOVA, E.G., DORONINA, N.V. AND TROTSENKO, Y.A. 2001, Aerobic methylobacteria are capable of synthesizing auxins. Mikrobiologiya, **70**: 345-347.

IVANOVA, E.G., DORONINA, N.V., SHEPELYAKOVSKAYA, A.O., LAMAN, A.G., BROVKO, F.A. AND TROTSENKO, Y.A., 2000, Facultative and obligate aerobic methylobacteria synthesize cytokinins. *Mikrobiologiya*, **69**: 764-769.

JACKSON, M.L., 1973, *Soil Chemical Analysis*, Prentice Hall of India (P) Ltd., New Delhi.

JANSEN, R., ROOD, S., DORMAR, J. AND MC-GILL, W., 1992, *Azospirillum brasilense* produces gibberellins in pure culture and chemically medium and in co-culture on straw. *Soil Biological Biochemistry*, **24**: 1061-1064.

JISHA, M.S. AND ALAGAWADI, A.R., 1996, Nutrient uptake and yield of sorghum inoculated with phosphate solubilizing bacteria and cellulolytic fungus in a cotton stalk amended vertisol. *Microbiological Research*, **151**: 213-217.

KATZNELSON, H. AND COLE, S.E., 1965, Production of gibberellin like substances by bacteria and actinomycetes. *Canadian Journal of Microbiology*, **9**: 733-741.

KEYSER, H.H. AND LIF, R., 1992, Potential for increasing biological fixation in soybean. *Plant and Soil*, **141**: 119-135.

KUNDU, B.S. AND GAUR, 1980, Establishment of nitrogen fixing and phosphate solubilizing bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. *Plant and Soil*, **57**: 223-230.

LEONG, J., 1986, Siderophores : Their biochemistry and possible role in the biocontrol of plant pathogens. *Annual Review of Phytopathology*, **24**: 187-209.

LIBBERT, E., WICHNER, S., SCHIEWER, U., RISCH, H. AND KAISER, W., 1966, The influence of epiphytic bacteria on auxin metabolism. *Planta*, **68**: 327-334.

LIDSTORM, M.E., 1992, In: *The Prokaryotes,* Eds. A. Balows , H.G. Truper, M. Dworkin, W. Harder and K.H. Schleifer, Springer Verlag, New York, pp. 431-445.

LINDERMAN, R.G., 1986, Rhizobacteria from field-grown mungbean isolation and identification. *Horticultural Science,* **21**: 1299-1302.

LONG, R.L., 2000, tRNA is the source of cytokinin secretion by plant-associated members of the genus *Methylobacterium. Ph.D Dissertation,* University of Missouri-Columbia.

MADHAIYAN, M., 2002, Molecular aspects, diversity and plant interaction of facultative methylotrophs occurring in tropical plants. *Ph.D. Thesis,* Tamil Nadu Agricultural University, Coimbatore.

MADHAIYAN, M., POONGUZHALI, S., LEE, H.S., HARI, K., SUNDARAM, S.P. AND SA, T.M., 2005a, Pink-pigmented facultative methylotrophic bacteria accelerate germination growth and yield of sugarcane clone Co86032 (*Saccharum officinarum* L.). *Biological Fertilizer Soils,* **41**: 350-358.

MADHAIYAN, M., POONGUZHALI, S., SUNDARAM, S.P. AND TONGMIN S.A., 2005b, A new insight into foliar applied methanol influencing phylloplane methylotrophic dynamics and growth promotion of cotton (*Gossypium hirsutum* L.) and sugarcane (*Saccharum officinarum* L.). *Environmental and Experimental Botany* (in press).

MADHAIYAN, M., POONGUZHALI, S., SENTHILKUMAR, M., SESHADRI, S., CHUNG, H., YANG, J., SUNDARAM, S.P. AND TONGMIN, S.A., 2004, Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp. *Botanical Bulletin Academy Sin*, **45**: 315-325.

MAHADEVAN, A. AND SRIDHAR, R., 1982, Hydrolytic enzymes. In: *Methods in Physiological Plant Pathology*, 2nd edition Sivakami Publishers, Madras, pp.24-44.

MAHADEVAN, A. AND SRIDHAR, R., 1986, In *Methods in Physiological Plant Pathology* (3rd Edn.), Sivakami Publications, Chennai, pp.9-11.

MEHTA, A.K. AND RAM MOHAN RAO, D.S., 1996, Effect of *Rhizobium* inoculation, nitrogen and phosphorus application on yield and yield attributes of groundnut. *Legume Research*, **19**: 151-514.

MUNSANJE, E., JOSHI, J., KITTEL, M., HOLLAND, M.A., 1996, Abstract, 6th Biennial conference on molecular and cellular biology of the soybean, University of Missouri, Columbia, MO.

MUROMTSEV, G.S., CHKANIKOV, D.I., KULAEVA, O.N. AND HAMBURG, K.Z., 1987, The hormonal regulation of physiological processes in flowering plants. *Osnovy Khimicheskoi Regulyatsii Rosta I Productivnosti Rastenii* (Basic Principles of the chemical regulation of plant growth and productivity), Moscow : Agropromizdat, pp.80-133.

NEILANDS, J.B. AND LEONG, S.A., 1986, Siderophores in relation to plant growth disease. *Annual Review of Plant Physiology*, **37**: 187-208.

NISHIJIMA, F., EVANS, W.R. AND VESPER, S.J., 1988, Enhanced nodulation of soybean by *bradyrhizobium* in the presence of *Pseudomonas fluorescens*. *Plant and Soil*, **111**: 149-150.

OBERHANSLI, T., DEFAGO, G. AND HAAS, D., 1991, Indole-3-acetic acid (IAA) synthesis is the biocontrol strain CHAO of *Pseudomonas fluorescence* ; Role of tryptophan side chain oxidase. *Journal of General Microbiology*, **137**: 2273-2279.

OMER, Z.S., TOMBOLINI, R., BROBERG, A. AND GERHARDSON, B., 2004, Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. *Plant Growth Regulator*, **43**: 93-96.

OSTLE, A.G. AND HOLT, J.G., 1982, Nile blue A as a fluorescent stain for poly β-hydroxybutyrate. *Applied Environmental Microbiology*, **44**: 238-241.

PARR, J.F., HORNICK, S.B. AND KAUFMAN, D.D., 1994, Use of microbial inoculants and organic fertilizers in agricultural production. In: *Proceedings of the International Seminar on the Use of Microbial and Organic Fertilizers in Agricultural Production*, Food and Fertilizer Technology Centre Publication, Taiwan, pp.13-18.

PATIL, A.B., 1992, Effect of combined inoculations on mineral nutrition and yield of leguminous crops. *Ph.D. Thesis*, State Agariarian University, St. Petersburg, Russia.

PATT, T.E., COLE, G.C., BLAND, J. AND HANSON, R.S., 1974, Isolation of bacteria that grow on methane and organic compounds as sole source of carbon and energy. *Journal of Bacteriology*, **120:** 955-964.

PATT, T.E., COLE, G.C., HANSON, R.S., 1976, *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *International Journal of Systemic Bacteriology*, **26**: 226-229.

PAULRAJ, A., 2002, Effect of azophos on raising cardamom seedlings under *in vitro* condition. *M.Sc.(Agri.) Thesis*, Tamil Nadu Agricultural University, Madurai.

PICCOLI, P., MASCIARELLI, O. AND BOTTINI, R., 1996, Metaboism of 17, 17-[$^2H_2$]-gibberellins A_4, A_9 and A_{20} by *Azospillum lipoferum* in chemically defined culture medium. *Symbiosis*, **21**: 263-274.

PICCOLI, P., MASCIARELLI, O. AND BOTTINI, R., 1999, Gibberellin production by *Azospirillum lipoferum* cultured in chemically defined medium as affected by oxygen availability and water status. *Symbiosis*, **27**: 135-145.

POI, S.C., GHOSH, G. AND KABI, M.C., 1989, Response of chickpea (*Cicer arietinum* L.) to combined inoculation with *Rhizobium*, phosphobacteria and mycorrhizal organisms. *Zentralblatt fur Mikrobidogie*, **144**: 249-253.

PRABHAKARAN, J., BALACHANDAR, D., NAGARAJAN, P. AND DHANAKODI, C.V., 1999, Effect of dual inoculation of *Rhizobium* and *Phosphobacteria* at different levels of phosphorus in horsegram. *Legume Research*, **22**: 137-138.

PRATHIBHA, C.K., ALAGAWADI, A.R. AND SREENIVASA, M.N., 1995, Establishment of inoculated organisms in rhizosphere and their influence on nutrient uptake and yield of cotton. *Karnataka Journal of Agricultural Sciences*, **8**: 22-27.

PRATHIBHA, C.K., ALAGWADI, A.R. AND SREENIVASA, M.N., 1995, Establishment of inoculated organisms in rhizosphere and their influence on nutrient uptake and yield of cotton. *Karnataka Journal of Agricultural Sciences*, **8**: 22-27.

QUAYLE, J.R., 1972, The metabolism of one-carbon compounds by microorganism. In: *Advances in Microbial Physiology*, Eds. Rose, A.H. and Tempest D.W., Academic Press Inoculation, New York, pp.119-203.

RAMARETHINUM, R., RAJAGOPAL, B., MARIMUTHU, S., 1998, Diazotrophic nature of the plant antagonistic bacterium *Bacillus subtilis*. *Pestology*, **22**(8): 46-50.

RANGASWAMI, G. AND BAGYARAJ, D.J., 1993, Microbial Biotechnology. In: *Agricultural Microbiology*, Prentice Hall of India Pvt. Ltd., New Delhi, pp.389-405.

RAO, L. AND DHIR, K.K., 1993, Some biochemical aspects on nitrogen fixation under salt stress in mungbean seedlings. In *New Trends in Plant Physiology, Proceedings of National Symposium on Growth and Differentiation in Plants.*

RAUT, M.S. AND MASOOD ALI, 1983, Response of clusterbean (*Cyamposis retragenaloba*) to phosphate application and rhizobial inoculation under dry land conditions. *Legume Research*, **6**: 65-68.

REEVES, M., NEILANDS, P.L. AND BALLOWS, A., 1983, Absence of siderophore activity in *Legionella* sp. grown in iron deficient media. *Journal of Bacteriology*, **154**: 324-329.

RICE, W.A., OLSEN, P.E. AND LESSET, M.E., 1995, Coculture of *Rhizobium* and phosphorus soluiblizing bacteria in sterile peat. *Soil Biological Biochemical*, **27**: 110-116.

SCHIFFMANN, J. AND LABEL, R., 1973, Seasonal changes in symbiotic nitrogen fixation and haemoglobin content in nodule of peanut. *Plant and Soil*, **39**: 329-340.

SEELEY, H.W. AND VANDEMARK, P.J., 1981, *Microbes in Action – A Laboratory Manual of Microbiology*, Freeman and Company, San Francisco, USA, p.388.

SENTHILKUMAR, M., 2003, Evaluating diazotrophic diversity and endophytic colonization ability *Azorhizobium caulinodans* and *Methylobacterium* sp. in bacterised and biotized rice (*Oryza sativa* L.). *Ph.D. Thesis*, Tamil Nadu Agricultural University, Coimbatore.

SENTHILKUMAR, M., MADHAIYAN, M., SUNDARAM, S.P. AND KANNAIYAN, 2002, Compatible nature of pink-pigmented facultative *Methylotrophs* with other bioinoculants. *Indian Journal of Microbiology*, **42**: 339.

SHEPELYAKOVSKAYA, A.O., DORONINA, N.V., LAMAN, A.G., BROVKO, F.A. AND TROTSENKO, YU, A., 1999, New data on the ability of aerobic methylotrophic bacteria to synthesize cytokinins. *Dokl Akad. Nauk*, **368**: 555-557.

SHOEF, T.W. AND LIUM, B.M., 1976, Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide, Limnol. *Oceangr*, **21**: 926-928.

SINGH, C.S., 1996, Arbuscular, mycorrhiza (AM) in association with *Rhizobium* spp. Improves nodulation, N_2 fixation and N utilization of Pigeonpea (*Cajanus cajan*) as a assessed with at 15 N technique, in pots. *Microbiological Research*, **151**: 98-92.

SIVAPRASAD, P. AND RAI, P.V., 1991, Syngergistic association between *Glomus fasciculatum* and *Rhizobium* sp. and its effect on Pigeonpea (*Cajanus cajan*). *Indian Journal of Agricultural Sciences*, **61**: 97-101.

SMIBERT, R.M., KREIG, N.R., 1981, General characterization. In: *Methodology for General Bacteriology*, Eds. P. Gerhardt, Academic Publisher, New York, pp.400-450.

SMITH, S.E. AND DRAFT, M.J., 1978, The effect of mycorrhizae on phosphate content, nitrogen fixation and growth of *Medicago sativa*. In: *Microbial Ecology*, Eds. M.W. Loutit and J.A.R., Millis, Springer Verlag, Heidelberg, pp.314-319.

SURESH REDDY, B.V., 2002, Studies on pink pigmented facultative methylotrophs as a new bioinoculant for groundnut (*Arachis hypogaea* L.). *M.Sc.(Agri.) Thesis*, Tamil nadu Agricultural University, Coimbatore.

SY, A., GIRUD, E., JOURAND, P., GARCIA, N., WILLEMS, A., LAJUDIE, P. DE., PRIN, Y., NEYRA, M., GILLS, M., CATHERINE, B.M. AND DREYFUS, B., 2001, Methylotrophic *Methylobacterium* bacteria nodulate and fix atmospheric nitrogen in symbiosis with legumes. *Journal Bacteriology*, **183**: 214-220.

TEIN, T.M., GASKINS, M.H. AND HUBBELL, D.H., 1979, Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Applied Environmental Microbiology*, **37**: 1016-1024.

THANGAMANI, G. AND SUNDARAM, S.P., 2005a, Effect of facultative *Methylobacterium* isolates on the hybrid rice (Co RH2) seed quality. *Abstract of Poster Presented in 3rd National Conference of Association of Applied Microbiologists (IAAM)*, 5-7, January 2005, Department of Microbiology, Adiprakasakthi College of Arts and Science, Kalava, Tamil Nadu, India, pp.63-64.

THANGAMANI, G. AND SUNDARAM, S.P., 2005b, Indole acetic acid production by facultative *Methylobacterium* sp. *Abstract of Poster Presented in 3rd National Conference of Association of Applied Microbiologists (IAAM)*, 5-7 January 2005, Department of Microbiology, Adiprakasakthi College of Arts and Science, Kalava, Tamil Nadu, India, p.65.

THANGAMANI, G. AND SUNDARAM, S.P., 2005c, Potential of facultative methylotrophs in increasing the yield of tomato crop. In: *Paper Presented in Proceedings of the ICAR National Symposium on Biotechnological Intervention for Improvement of Horticultural Crops : Issues and Strategies in Symbiohort*, 10-12 January, 2005 Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Thrissur, Kerala, pp.362-365.

THANGAMANI, G., 2005, Studies on facultative methylotrophs for increasing crop production. *Ph.D. Thesis*, Tamil nadu Agricultural University, Coimbatore.

URAKAMI, T. AND KOMAGATA, K., 1984, *Protomonas*, a new genus of facultatively methylotrophic bacteria. *International Journal Systemic Bacteriology*, **34**: 188-201.

WHITTENBURY, R., DAVIES, S.L. AND WILKINSON, J.F., 1970, Enrichment, isolation and some properties of methane utilizing bacteria. *Journal of Gen Microbiology*, **61**: 205-218.

Appendices

APPENDIX I

Composition of media

Ammonium mineral salt medium (AMS)

NH_4Cl	:	0.50 g
K_2HPO_4	:	0.70 g
KH_2PO_4	:	0.54 g
$MgSO_4.7H_2O$	:	1.00 g
$CaCl_2.2H_2O$	:	0.20 g
$FeSO_4.7H_2O$	:	4.00 mg
$ZnSO_4.7H_2O$	:	100.0 µg
$MnCl_4.4H_2O$	:	30.0 µg
H_3BO_4	:	300.0 µg
$CoCl_2.6H_2O$	:	200.0 µg
$CuCl_2.2H_2O$	:	10.0 µg
$NiCl_2.6H_2O$	:	20.0 µg
$Ha_2MoO_4.2H_2O$	:	60.0 µg
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	6.8
Methanol	:	0.5%

N-free malate medium

Malic acid	:	5.0 g
Dipotassium hydrogen orthophosphate	:	0.5 g
Magnesium sulphate	:	0.2 g
Sodium chloride	:	0.1 g
Calcium chloride	:	2.0 g
Fe-EDTA (1.64% w/v aqueous)	:	4.0 ml
Trace element solution	:	2.0 ml
Bromothymol blue (0.5% alcoholic solution)	:	2.0 ml
Vitamin solution	:	1.0 ml
Potassium hydroxide	:	4.0 g
Agar (Semi solid)	:	1.75 g
Distilled water	:	1000s ml
pH	:	6.8

Vitamin solution

Biotin	:	10.0 mg
Pyridoxin	:	20.0 mg
Distilled water	:	1000 ml

Tryptone glucose broth

Tryptone	:	1.0 g
Glucose	:	1.0 g
Distilled water	:	100 ml

Skim milk agar

Skim milk powder	:	100.0 g
Peptone	:	5.0 g
Agar	:	15.0 g
pH	:	7.2

MR-VP Broth (pH 6.9)

Peptone	:	7.0 g
Dextrose	:	5.0 g
Potassium phosphate	:	5.0 g

Simmon's citrate agar

Sodium citrate	:	0.2 g
Magnesium sulphate	:	0.02 g
Ammonium dihydrogen Phosphate	:	0.1 g

Czapadox mineral salt agar medium

Sodium nitrate ($NaNO_3$)	:	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

Hathaway 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.

Salper's reagent

0.5 M $FeCl_3$	:	1.0 ml
35% $HClO_4$	:	50 ml

Reagents for spectrophotometric estimation of GA

- **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.

- **Potassium ferrocyanide solution**: 1.6 g of potassium ferrocyanide solution in 100 ml of distilled water.

APPENDIX II

Physico-chemical properties of soil

Particulars	Value obtained	Procedure employed
Physical properties (%)		
Coarse sand (%)	7.23	
Fine sand (%)	11.20	International Pipette method (Piper, 1966)
Silt (%)	13.75	
Clay (%)	66.85	
Chemical properties		
Soil reaction (pH)	6.0	Systronics pH meter (Jackson, 1973)
Electrical conductivity (dS/m)	0.24	Wheat Stone Bridge (Jackson, 1973)
Organic carbon (%)	0.85	Wet oxidation method (Jackson, 1973)
Available N (kg/ha)	450	Alkaline potassium permanganate method (Subbaiah and Asija, 1959)
Available P_2O_5 (kg/ha)	45	Olsen's method (Jackson, 1973)
Available K_2O (kg/ha)	130	Flame photometer method (Jackson, 1973)
Available Grigg's MO (ppm)	0.20	Colorimetric method (Johanson and Arkley, 1954)
DTPA extractable micronutrients		
DTPA-Zn (ppm)	0.80	Atomic absorption spectrophotometer method (Lindsay and Norwell, 1978)
DTPA-Fe (ppm)	10.0	

STUDIES ON METHYLOTROPHS AND THEIR BENEFICIAL EFFECTS ON SOYBEAN (*Glycine max* (L.) Merrill)

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ABSTRACT

Investigations were carried to study the Methylotrophs and their beneficial effects on soybean. Samples were collected from Dharwad and Belgaum districts of Karnataka; about forty isolates were isolated on selective AMS medium. Further the isolates were characterized based on their morphological and biochemical characteristics. The results revealed that all the isolates were Gram negative, rod shaped with the dimension of 0.6-1.0 x 1.0-1.5 μm, accumulate PHB, colonies were pink, pale pink or dark pink in colour due to pigmentation. Biochemical characteristics revealed that all the isolates showed positive results for oxidase test, urease test, indole production. None of the isolates showed positive results for casein hydrolysisn, hydrogen sulphide production, MR and VP test, nitrate reduction test, all the isolates showed positive results for utilization of glucose, D-galactose, dichloromethane, chlorofrom and glycerol.

Further the isolate were screened for beneficial characters. The results revealed that the highest Indole acetic acid production was recorded in ML_{55} (28.15 μg/ml of culture filtrate) the maximum GA production of 70.27 μg/ml of culture filtrate was observed in ML55, the highest nitrogen fixation was observed in ML_2 (1.187 mg N/g of malate medium). The isolates were also tested for cytokinin production, siderophore production and P-solubilization; the results revealed that the maximum zeatin production was recorded in ML_2 (10.1 ng/ml of culture filtrate). The maximum siderophore production as recorded in ML_2 (0.60 μ moles of α-2, 3 di hydroxy benzoic acid). But none of the isolates were able to solubilize phosphate. Based on these beneficial characters three efficient isolates were selected for pot culture experiment along with *Bradyrhizobium japonicum* strain SB_{120}. Results revealed that compared to single inoculation treatment combined inoculation treatment performed better, among combined inoculation treatment T_9 (Reference strain + *B. japonicum* strain SB_{120} and T_7 (ML_{55}+*B. japonicum* strain SB_{120}) recorded higher growth and yield parameters.