

**PERFORMANCE OF METHYLOTROPHS IN SOYBEAN
(*Glycine max* (L.) Merrill) UNDER FIELD CONDITIONS**

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By

MEENAKSHI B. C.

**DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD - 580 005**

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3.

(B. BASAVARAJ)

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1. INTRODUCTION

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. a pink pigmented facultative methylotroph (PPFM) which was 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 on 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 methylotroph (PPFM) and non-pigmented facultative methylotroph (NPFM) bacteria which are capable of growing on C1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substates such as C2, C3 and C4 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 on actively growing and meristamatic tissue, sometimes averaging 10^4 colony forming units (cfu) per leaflet (Corpe, 1985; Dunleavy, 1988; Corpe and Rheem, 1989; Hirano and Upper, 1992; Holland and Polacco, 1992, 1994; Chanaprarn *et al.*, 1996; Holland, 1997).

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 Polacco; 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 an important legume and oil seed crop indigenous to China and introduced to India in 1950's. In the world it occupies 71.85 million ha with a production of 154.32 million tonnes annually. In India, it is grown over an area of 7.74 million ha with a production of 8.27 million tonnes (Anon., 2006). The crop is well suited to black soil and in Karnataka, it is grown as a major oil seed crop in Belgaum, Dharwad, Bagalkot and Haveri districts occupying 125 million ha of area with an annual production of 96 million tonnes (Anon., 2005). The golden bean contains the highest lysine rich protein (43.2%) among the leguminous crops with 19.5 per cent of oil containing fairly high amount of unsaturated fatty acids (Gopalan *et al.*, 1994). Soybean (*Glycine max* (L) Merrill) is a fascinating crop with innumerable possibilities of not only improving agriculture but also supporting industries.

The rhizobia that nodulate soybean were grouped as *Bradyrhizobium japonicum* (Jordon, 1982) that forms symbiotic association with soybean to fix atmospheric nitrogen and, in turn, gets photosynthates from the macrosymbiont. In the presence of the efficient *Bradyrhizobium* strain under favourable conditions, the crop not only meets its nitrogen requirement by fixing 150-300 kg N per ha per annum but also ensures considerable amount of residual nitrogen for subsequent crops (Puppepke and Zdor, 1987). The effective symbiosis helped to increase the grain yield to an extent of 50 percent when inoculation was done with efficient *Bradyrhizobium* strain in 'N' deficient soils.

The concept of active management of rhizosphere population of legumes has advanced towards co-inoculation of *Rhizobium/Bradyrhizobium* strains with plant growth promoting rhizobacterial strains (PGPR) as adjunct inoculants. These bacteria stimulate the growth of important crop plants under field conditions. Combined use of two or three beneficial microorganisms as inoculation have been found to perform better than single inoculations (Patil *et al.*, 1992; Alagawadi and Gaur, 1998; Jisha and Alagawadi, 1996; Prathibha *et al.*, 1995). Besides they exert a more accent effect. Hence, the combined inoculations are better than single inoculations (Patil *et al.*, 1992).

Combined use of plant growth promoting rhizobacteria is based on the principles of natural ecosystems, which are sustained by their constituents, that is, by the quality and quantity of their inhabitant and specific ecological parameters *i.e.*, the greater the diversity and number of inhabitants, the higher the order of their interaction and more stable the ecosystem. This concept of combined use of plant growth promoting rhizobacteria is an effort

to shift microbiological equilibrium in favour of increased plant growth production, nutrient uptake and protection (Higa, 1991 and Parr *et al.*, 1994). 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, yield and nutrient uptake in soybean with the following objectives.

1. To study the effect of inoculation of methylotrophs on growth and yield parameters in soybean under field conditions
2. To study the effect of inoculation of methylotrophs on nutrient uptake in soybean
3. To study the population dynamics of methylotrophs and other beneficial organisms and enzyme activities

2. REVIEW OF LITERATURE

Bradyrhizobium japonicum nodulates soybean (*Glycine max* (L) Merrill). It is a slow growing bacterium and fixes atmospheric nitrogen in symbiotic association with soybean to an extent of 300kg N/ha (Bezdicsek *et al.*, 1978).

Availability of nitrogen is considered to be a major limiting factor for dry matter and yield production in grain legumes. Biological nitrogen is one of the major sources of nitrogen for plant growth and seed yield in legume crops (Bushby *et al.*, 1983 and Gaur, 1993). So concentrated attempts are being made for increasing the yield of leguminous crops by proper techniques of inoculation to supplying the nutrients to the soil (Mehrotra and Gangawar, 1964; Mehrotra and Lehri, 1970).

When inoculated, rhizobia colonize the rhizosphere and enhance the plant growth by providing it with nitrogen (Kundu and Gaur, 1980). Whereas PPFMS colonize rhizosphere 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. They are a substantial part of the aerobic, heterotrophic micro flora of the surfaces of young leaves. These bacteria are capable of growth on C₁ compounds like methanol and methylamine and also on a variety of C₂, C₃ and C₄ 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 as an essential feature.

2.2 EFFECT OF INOCULATION OF METHYLOTROPHS ON GROWTH AND YIELD OF SOYBEAN

2.2.1 Seed germination and seedling establishment

The ubiquitous epiphytic species of genus *Methylobacterium* play a major role in the promotion of seed germination and seedling growth (Holland, 1997).

Sundaram *et al.* (2002) stated that PPFMs influence seed germination and seedling growth by producing the plant growth regulator zeatin and related cytokinins. Due to the production of plant hormone and vitamins by pink pigmented facultative methylotrophic bacteria colonizing on the phyllosphere regions of maize, they enhanced the seedling vigour. The application of PPFMs to the rice seedlings increased the effective utilization of urea which ultimately improved the metabolic activity of crops (Madhaiyan, 2002).

Lee *et al.* (2006) reported that methylotrophic strains promoted germination and growth of rice seedlings. Treated seeds recorded increased growth rate over control with a mean of 20.51 per cent and a range from 22.17 to 22.69 per cent. The results suggests the potential use of these bacteria to stimulate germination, seedling vigour index and biomass production, which is mediated by production of plant hormone accumulation and nitrogen fixation.

M. extroguense ME₄ isolated from *Fragaria xananassa* promoted the growth of various plant seedlings. Seedling weight and shoot length of *Nicotiana tabacum*, *Lycopersicon esculentum*, *Sinapsis album* and *Fragaria verca* were increased in the presence of the PPFM (Abunda-Nkpwatt *et al.*, 2006).

2.3 COMBINED INOCULATION OF *B. JAPONICUM* AND OTHER PGPR ON CROP PLANTS

Combined use of two groups of beneficial organisms helps in improving the efficiency of inoculated organisms (Alagawadi, 1986).

Alagawadi (1986) and Alagawadi and Gaur (1988) observed significant increase in nodule nitrogenase activity, nutrient uptake, plant biomass and grain yield of chickpea due to combined inoculation of rhizobium and phosphate solubilizing *Pseudomonas striata* or *B. polymxa* as compared to single inoculation treatments.

Increase in dry matter production and phosphate uptake in chickpea plant due to triple inoculation with rhizobium phosphobacteria and *Glomus fasciculatum* was reported (Poi *et al.*, 1989).

Savalgi *et al.* (1994) recorded higher nodulation due to dual inoculation with *Azospirillum brasilense* strains and rhizosphere spp. strain NC-92 than *Rhizobium* alone in groundnut under red loam soil condition. Between *Azospirillum* isolates ACR-8 (local isolate) showed better performance than SP-7.

Patel *et al.* (1998) showed combination of 50 per cent N and P coupled with *Rhizobium* and phosphate solubilizing microorganisms increased plant height, number of branches, leaves/plant, number of pods/plant, grains/pod and pod yield significantly in garden pea compared to recommended levels of nutrients applied through chemical fertilizers only.

Nagaraj (2002) isolated and characterized PGPR such as *Rhizobium*, *Azospirillum* and phosphate solubilizers for their beneficial traits such as N₂-fixation, phosphate solubilization, production of PGP substances such as IAA, GA and biocontrol activity. After considering all these beneficial traits, one efficient strain was selected to study the combined effect of these growth promoting rhizobacteria on the growth and yield of soybean.

2.4 CO-INOCULATION OF METHYLOBACTERIUM AND OTHER PGPR ON PLANT PRODUCTIVITY

Holland and Polacco (1992) reported a method for increasing productivity of soybean by spraying pink pigmented facultative methylotrophic (PPFM) bacteria on a plant. The invention also relates to increasing productivity of the plant under stress by applying 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 (Musanje *et al.*, 1996). In field trials, 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 measurement 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 (Musanje, 1996). PPFMs could be used as seed inoculum (or) in seed coatings designed to enhance germinability, storability (or) vigour of the seeds.

Balamurugan and Gunasekaran (1996) found maximum number of nodule per plant and higher nutrient content with combined inoculation of *Rhizobium*, *Azospirillum* and phosphobacteria.

Holland (1997) and Ramarethinam and Rajagopal (1998) 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, 2005).

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 (2005) 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.

Kalyaeva *et al.* (2003) found the effects of four aerobic methylophilic bacteria on the morphogenesis of soft wheat (*Triticum aestivum*) under *in vitro* using immature embryos as explant. The inoculation of the embryos with methylophilic bacteria led to their stable colonization with the bacteria. The colonization of the explants with the strains of *Methylobacterium* sp. D10 and *Methylophilus lucoseoxidans* stimulated the formation of morphogenic calli and shoots and also promoted development of the regenerated plants. These regenerated plants manifested bright green leaves and a well developed root system. The colonization of immature wheat embryos with methylophilic bacteria can be employed as a tool for raising the efficiency of genetic transformation of various wheat cultivars.

Madhaiyan *et al.* (2004) reported that the plant canopy provides an excellent niche for the establishment and the multiplication of a wide range of microorganisms. Among the bacterial genera on the plant surface, the pink pigmented facultative methylophilic (PPFMs) are associated with seeds and leaves of all the tropical and temperate crops. They are present ubiquitously on the plant canopy and play a role in the production of cytokinins and vitamins. An attempt was made to study the effect of inoculation of ten PPFM strains on germination and the growth of soybean. The soybean seeds were treated with PPFM. Strains and tested under pot culture condition. Results suggested that the *Methylobacterium* sp. (Sb 34) isolates from soybean increased the shoot length (26.4 cm plant⁻¹), root length (25.01 cm plant⁻¹), plant biomass (11.25 g fresh weight plant⁻¹ and 2.05 g dry weight plant⁻¹) and nodulation efficiency (13.5%) followed by *Methylobacterium* sp. obtained from black gram recording shoot length (25.4 cm plant⁻¹), root length (23.22 cm plant⁻¹), plant biomass (10.25 g fresh weight plant⁻¹ and 2.01 g dry weight plant⁻¹) and nodulation efficiency (12.5%) in soybean. The increased plant parameters may be attributed to the production of cytokinins and vitamins by pink-pigmented facultative methylophilic bacteria.

Madhaiyan *et al.* (2005) reported that the application of PPFMs as a foliar spray significantly increased plant height, plant dry weight, boll number, boll weight and kapas yield of cotton. Thangamani and Sundaram (2005) confirmed that the facultative methylophilic have a greater importance in increasing the growth and yield of various crop plants.

Madhaiyan *et al.* (2005) revealed that inoculation of PPFMs resulted in a significant increase in sugarcane plant growth, cane yield and sugar quality. The presence of non-pigmented facultative methylophilic in roots of greengram (*Vigna radiata* L.) was well documented by Senthilkumar *et al.* (2002).

Sugarcane true seeds inoculated with PPFMs had a higher germination rate than the control. A combined treatment of seed imbibition, soil application and phyllosphere spray increased specific leaf area, plant height, number of internodes and cane yield. Immunological determination showed that cytokinin in young and mature leaves significantly increased when the epiphytic population on the leaf surface increased (Madhaiyan *et al.*, 2005).

Facultative methylophilic were found to be compatible with nitrogen fixing *Azospirillum* sp., Phosphate solubilizing *Pseudomonas* sp. and other bioinoculants. Dual inoculation of PPFM and *Azospirillum* sp. Showed significant influence in the early development of cotton. The combined application of PPFM and *Bacillus* sp showed major role in the later stage of crop growth like flower and boll development (Senthilkumar and Sundaram, 2005).

Madhaiyan *et al.* (2005) studied the effect of pink-pigmented facultative methylophilic bacteria on germination, growth and yield of sugarcane clone CO 86032. 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 percent 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.

Field trials and pot culture studies of cotton showed that application of 30 per cent methanol (or) PPFMs as foliar spray significantly increased plant height, leaf area, boll number and boll dry weight, leading to increase of seed cotton yield (SCY) over control. Foliar

application of PPFMs increased plant height and specific leaf area of sugarcane and led to a cane yield increase of 9.8 per cent over control. Application of methanol (or) PPFMs increased the total cytokinin in cotton and sugarcane (Madhaiyan *et al.*, 2006b).

Lee *et al.* (2006) reported that methylotrophic strains promoted germination and growth of the rice seedlings. Treated seeds recorded increases in growth rate over control with a mean of 20.51 per cent and a range from 22.17 to 22.69 per cent. The results suggests the potential use of these bacteria to stimulate germination, seedling vigour index and biomass production, which is mediated by production of plant hormone accumulation and nitrogen fixation.

Madhaiyan *et al.* (2006a, 2007) reported the presence of ACC deaminase in *Methylobacterium fujisawaense* and *Myc oryzae* and its lowering of ethylene levels and promotion of root elongation in canola seedlings under gnotobiotic conditions. Lower amounts of ACC were present in the tissues of seeds treated with *M. fujisawaense* strains than in control. Low levels of ethylene appear to enhance root extension; higher levels of ethylene, produced by fast growing roots, can lead to inhibition of root elongation (Mattoo and Suttle, 1991; Ma *et al.*, 1998).

Thangamani and Sundaram (2007) reported that microbial consortia of PPFM, *Azospirillum* sp., *Azotobacter* sp. PSB and VAM were found to increase the yield of tomato crop by 62.73 per cent over uninoculated control. Combined inoculation of *Methylobacterium* sp. with *Rhizobium* sp. also significantly increased plant growth, nodulation and yield attributes in groundnut compared with individual inoculation of *Rhizobium* sp., *Methylobacterium* sp. (Madhaiyan *et al.*, 2006a&b). Methylotrophs have received a great deal recently due to their importance in the biosphere and their potential commercial applications.

2.5 POPULATION DYNAMICS OF METHYLOTROPHS

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

Freyermuth *et al.* (1996) reported that seed germination and development of plants treated with PPFM's and PPFM spent medium showed, inoculated plants performing better than the uninoculated. Reduction in the population of PPFMs in the seed coat also led to reduce germination levels speculating the role of PPFMs on seed germination. The population of PPFMs ranged from 10^5 to 10^9 cfu g⁻¹ fresh tissue and they could not be dismissed as accidental visitors on 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, facultative methylotrophic bacteria (PPFMs) are normally associated with seeds and leaves of soybean.

Senthilkumar *et al.* (2002) studied the compatibility of a pink pigmented facultative methylotroph. 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, *Pseudomonas fluorescens* PF1, *Trichoderma viridae* 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.* (2004) stated that PPFMs are ubiquitous in nature and found in a variety of habitats. They made an attempt to isolate the PPFMs from phyllosphere of cereals, pulses, oilseeds, vegetables, fruit crops, species, commercial crops, flower crops. Among the different crops tested, the maximum population of PPFMs was found in the phyllosphere. The occurrence of PPFMs were comparatively lesser in *Carcia papaya* and *Cucumis sativus*.

Schauer and Kutschera (2008), reported that plant associated methylotrophs of the genus *methylobacterium* colonized the foliage and roots of embryophytes, living on the volatile compound methanol emitted from the cells of their host organism. In this study, they

analyzed the surface dwelling pink pigmented epiphytes in three contrasting habitats of field grown sunflower plants (*Helianthus annuus*) using the methanol ammonium salts agar surface impression method and a polymerase chain reaction (PCR) based assay. They documented their occurrence and characterized the composition of the methylobacteria in these epiphytic habitats. In both the sun-exposed phylloplane and the moist, dark rhizoplane, pink-pigmented methylobacteria were detected that were assigned to the taxa *M. mesophilicum*, *M. extorquens*, *M. radiotoleran* and *M.sp.*

2.6 EFFECT OF PGPR ON NUTRIENT UPTAKE STUDIES

Kundu and Gaur (1980) reported an increased wheat yield due to inoculation of *Azotobacter chroococcum*, *B. polymyxa* and *Pseudomonas striata* as combined inoculum over their single inoculations. The combined inoculations positively influenced the nitrogen and phosphorus uptake of plants and also noticed higher microbial populations of both nitrogen fixing and phosphate solubilizing bacteria in the mixed inoculations indicating possible synergistic interaction between the two groups.

Alagawadi and Gaur (1988) reported that inoculation of *Rhizobium*, *P.striata* or *B. polymyxa* significantly increased the nitrogen and phosphorus uptake by chickpea over control. The uptake was further enhanced with the application of 10kg N and 60 kg P₂O₅ per ha. The highest N and P uptake was recorded in *Rhizobium* + 20 kg N and 60 kg P₂O₅ per ha.

Parvatham *et al.* (1989) reported that *Azospirillum* inoculation improved nitrogen and phosphorus availability in the rhizosphere of bhendi crop thereby facilitating better uptake of nitrogen and phosphorus in black clay soil.

Hyoung *et al.* (2006) stated that three plant growth promoting, N₂ fixing methylotrophic strains were isolated from rice cultivars (*Oryza sativa* L.), viz., *Methylobacterium* sp. CBMB 20, *Enterobacter* sp. CBMB 30, *Burkholderia* sp. CBMB 20. Their activities in promoting the early growth of rice were studied. Seeds treated with the methylotrophic strains improved seed germination, seedling vigour index (SVI) and biomass of rice seedlings. The results suggest the potential use of these bacteria to stimulate germination, SVI and biomass production, which is mediated by production of plant hormone accumulation and nitrogen fixation.

2.7 ENZYME ACTIVITIES

Enzymes in the soil are biologically significant as they participate in various transformations and influence the availability of plant nutrients. In soil microorganisms, active roots and dead cells are the principal sources of enzymes. A major proportion of the extra cellular enzymes found in soil get adsorbed on to clay and humic colloids.

Bolten *et al.* (1985) reported that treatment which received nitrogen from continuous leguminous green manuring crops had significantly higher level of dehydrogenase, urease, phosphatase activity and also biomass compared to the treatment received the NPK fertilizers for 30 years.

Singaram and Kamalakumari (1995) in a long term experiment studied six soil enzymes related to carbon, nitrogen and phosphorus cycling. The enzyme such as amylase, catalase, cellulase, dehydrogenase, phosphatase and urease were found to be superior in the FYM treatment.

Raj *et al.* (2004) studied the effect of application of compost enriched with microbial consortium (*Aspergillus-Pleurotus-Trichoderma*) in tomato plants. The results revealed that total tomato fruit weight was increased (1.2 kg/bag) compared to only compost (0.86 kg) and normal method (0.43 kg) without compost. And, they observed the enhancement of microbial population, enzyme activity and microbial biomass C, N and P at 60th day after transplanting.

3. MATERIAL AND METHODS

The present investigations were carried out during rabi, 2007-08 at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad to evaluate the performance of methylotrophs in soybean crop under field conditions. The details of the material used and method followed for the present study are discussed in this chapter.

3.1 LOCATION

The present study was conducted in field No. 26A E- Block plot of MARS University of Agricultural Sciences, Dharwad, which is situated in the transition tract of Karnataka state at 15°26' north latitude, 75° 71' east longitude and at an altitude of about 678 meters above the mean sea level.

3.2 SOIL CHARACTERISTICS OF EXPERIMENTAL SITE

The soil of the experimental site was medium black clay in nature. Composite soil samples upto a depth of 30 cm were collected from the experimental site, before initiating the experiment and analyzed for important physical and chemical properties as well as microbial population by employing standard methods. The results are presented in Table 1.

3.3 AGRO-CLIMATIC CONDITIONS

The data on climatic parameters such as rainfall, maximum and minimum temperature and relative humidity recorded at Meteorological Observatory, Main Agricultural Research Station, Dharwad during experimental year 2007-08 till the completion of experiment are presented in Appendix I.

3.4 PREVIOUS CROP ON THE EXPERIMENT SITE

During 2005-06 *rabi* sorghum was grown on the experimental site.

Isolates used in the present study

The methylotrophic bacterial isolates *viz.*, ML2 isolated from the phyllosphere of chilli, ML55 isolated from the rhizosphere of sugarcane, ML66 isolated from the phyllosphere of tomato crop and maintained in culture bank of Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad for the present study to know the efficiency of test isolates under field conditions on soybean.

Preparation of the inoculum

Five hundred g of lignite powder was neutralized to adjust the pH and mixed with CaCO₃ thoroughly. The properly mixed lignite powder was packed in sterilizable polypropylene (PP) bags and sterilized in an autoclave at 121 °C for an hour.

3.5 INOCULATION TO THE CARRIER

The bioinoculants were prepared by inoculating 72 h old log phase culture of strains of *Methylobacterium* in 100 ml of AMS broth (Whittenbury *et al.*, 1970) and *Bradyrhizobium* (Strain SB₁₂₀) in 100 ml of YEM broth. The flasks were kept on a shaker at 28±2°C for five days. The culture broth was then mixed with pre-sterilized lignite powder @ 15 ml of *Methylobacterium* isolate and 15 ml of *Bradyrhizobium* strain SB₁₂₀ per 30 g of carrier. The foliar spray was taken with *Methylobacterium* isolates at 20, 30 and 45 DAS in 1:100 dilution and sprayed @ 50 ml per plot. The treatments were as follows.

- T₁ – Seed inoculation with ML₂ and *B. japonicum*
- T₂ – Seed inoculation with ML₅₅ and *B. japonicum*
- T₃ – Seed inoculation with ML₆₆ and *B. japonicum*
- T₄ – Seed inoculation with Reference strain and *B. japonicum*
- T₅ – Seed inoculation with *B. japonicum* (Control)
- T₆ – T₁ + foliar spray with ML₂
- T₇ – T₂ + foliar spray with ML₅₅
- T₈ – T₃ + foliar spray with ML₆₆
- T₉ – T₄ + foliar spray with Reference strain

Table 1. Physico-chemical and biological properties of soil

Sl. No.	Particulars	Value obtained	Procedure employed
I	Particle size analysis		International Pipette method (Piper, 1966)
1	Coarse sand (%)	7.25	
2	Fine sand (%)	14.25	
3	Silt (%)	28.70	
4	Clay (%)	51.00	
5	Texture	Clay	
II	Chemical properties		
1	Soil reaction (pH)	8.00	Systronics pH meter (Jackson, 1973)
2	Electrical conductivity (dS/m)	0.23	Wheat stone bridge (Jackson, 1973)
3	Organic carbon (%)	0.57	Wet oxidation method (Jackson, 1973)
4	Available N (kg/ha)	170.00	Alkaline potassium permanganate method (Subbaiah and Asija, 1959)
5	Available P ₂ O ₅ (kg/ha)	20.75	Olsen's method (Jackson, 1973)
6	Available K ₂ O (kg/ha)	304.59	Flame photometer method (Jackson, 1973)
III	Initial microbial population		
1	Bacteria (cfu/g dry soil)	78.58x10 ⁶	Dilution plate technique
2	Fungi (cfu/g dry soil)	31.60x10 ³	Dilution plate technique
3	Actinomycetes (cfu/g dry soil)	10.26x10 ³	Dilution plate technique

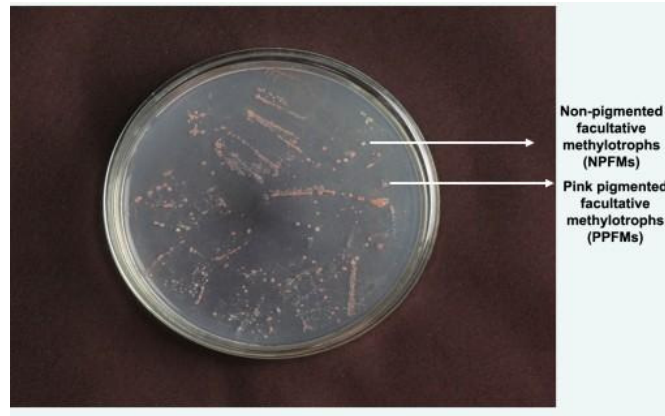


Plate 1. Growth of pink pigmented facultative methylotrophs on AMS medium



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

Table 2. Details of field experiment

Sl. No.	Particulars	Details
1	Cultivar of soybean	JS-335
2	Design	Randomized Block Design
3	Treatments	T ₁ – Seed inoculation with ML ₂ and <i>B. japonicum</i>
		T ₂ – Seed inoculation with ML ₅₅ and <i>B. japonicum</i>
		T ₃ – Seed inoculation with ML ₆₆ and <i>B. japonicum</i>
		T ₄ – Seed inoculation with Reference strain and <i>B. japonicum</i>
		T ₅ – Seed inoculation with <i>B. japonicum</i> (Control)
		T ₆ – T ₁ + Foliar spray with ML ₂
		T ₇ – T ₂ + Foliar spray with ML ₅₅
		T ₈ – T ₃ + Foliar spray with ML ₆₆
		T ₉ – T ₄ + Foliar spray with Reference strain
4	Replication	Three
5	Plot size	
	Gross plot	3 m x 2.7 m (8.1 mt ²)
6	Layout	Randomized Completely Block Design (RCBD)
7	Spacing	30 cm x 10 cm
8	Fertilizer (NPK kg ha ⁻¹)	40:80:25 N, P ₂ O ₅ , K ₂ O kg ha ⁻¹

3.6 REFERENCE STRAIN CULTURE COLLECTION

The reference culture *Methylobacterium extorquens* (ME-14718) was obtained from American Type Culture Collection (ATCC) and used.

3.6.1 Land preparation and layout

The experimental site was brought to fine tilth by ploughing once with tractor iron plough and was followed by two harrowing. The experiment was laid out as per the plan given in Fig. 1 and the plots were provided with bunds all around to avoid contamination of soil and water from other plots. Irrigation channels were also made to facilitate irrigation.

3.6.2 Fertilizer application

The recommended dose of 40:80:25 kg N: P: K per ha for soybean crop was applied in the form of urea, single super phosphate and murate of potash respectively as basal dose to each plot and FYM was applied at the rate of 7 t/ha at the time of sowing.

3.6.3 Seeds and sowing

The soybean variety JS-335 obtained from AICRP on Soybean, Main Agricultural Research Station, University of Agricultural Sciences, Dharwad was used for sowing.

Before sowing, seeds were treated with *Methylobacterium* strains and *Bradyrhizobium* biofertilizers @ 500 g per ha (10^9 cells/ml), as per treatment schedule. Seeds were sown in each plot and after germination thinning was done to maintain the required plant population. Three replications were maintained for each treatment. Necessary plant protection measures were taken up as per the recommended practices

Samplings were made at 30, 60 days after sowing and at harvest for maturity, growth, nutrient uptake and enzyme activities in soil. The plants were uprooted without disturbance to the roots and immediately washed with tap water. The plant samples were air dried for a week and then in a 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 the powdered plant samples were preserved for further elemental analysis.

3.6.4 Foliar spraying of *Methyl bacterium* isolates

The *Methylobacterium* cultures broth based (1×10^9 cfu ml⁻¹ of culture) was diluted to 1:100 and sprayed @ 50 ml/plot.

3.7 OBSERVATIONS ON GROWTH AND YIELD PARAMETERS

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

3.7.1 Growth parameters

Plant height

The plant height of the five randomly selected plants was measured from the base of the plant to the growing tip and their mean was expressed in cm.

Number of leaves per plant

The number of fully opened trifoliate leaves present in each of the five plants was counted and their mean was taken as the number of leaves per plant.

Number of branches per plant

Total number of branches per plant in each of the five plants was counted and the average was recorded as number of branches per plant.

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.

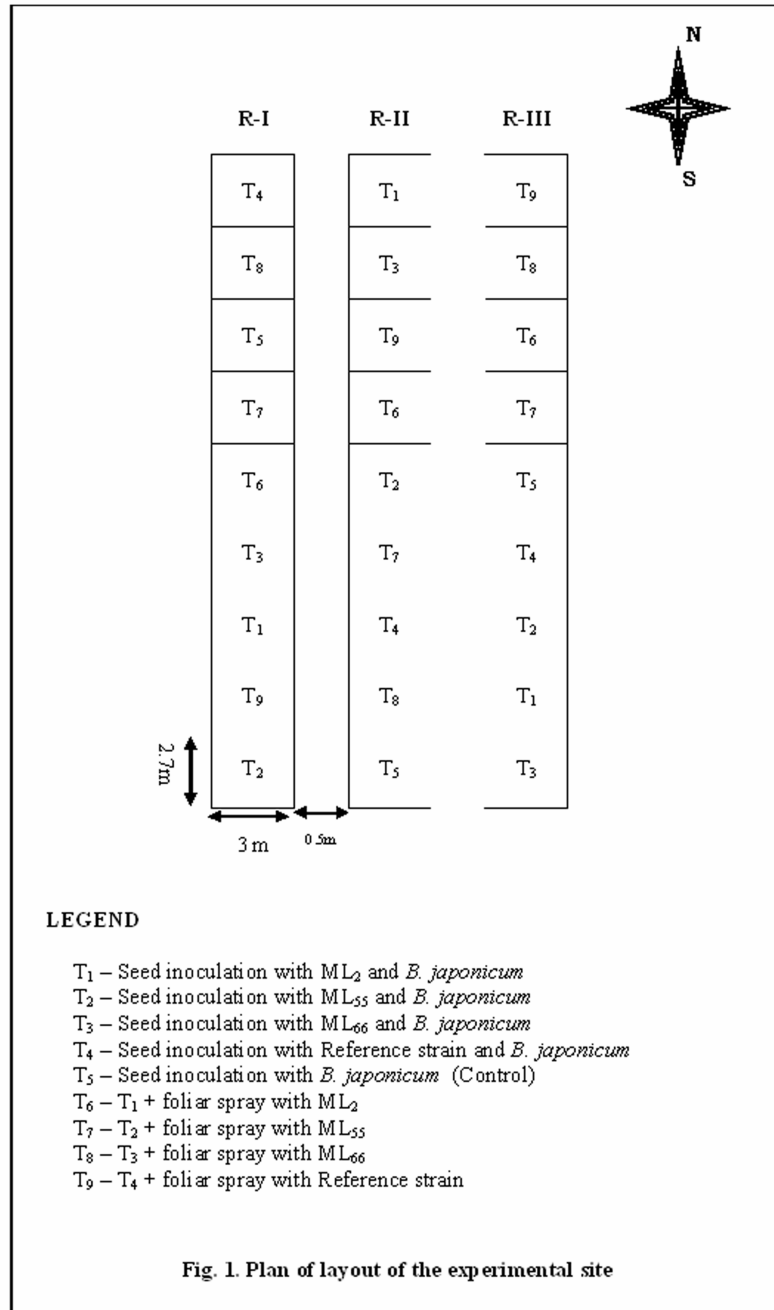


Fig. 1. Plan of layout of the experimental site



Plate 3. General view of experimental plot

Plate 3. General view of experimental plot

Nodule dry weight

Nodules were separated from the root system and oven dried to get 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).

Dry matter accumulation

The randomly selected five plants from sampling area were partitioned as stem, leaves and pods and dried in hot air oven at 65°C and dry weight was recorded separately after obtaining the constant dry weight. The sum of the mean dry weight of all the plant parts was taken as the total dry matter production per plant and expressed in grams per plant (g/plant).

3.7.2 Yield parameters

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 harvest. .

Test weight of grains

Hundred seeds were counted from the sample collected randomly from the net plot yield and their weight was taken as hundred seed weight and expressed in grams.

Grain yield

Yield of soybean seeds per net plot was recorded (kg/plot) and finally expressed in quintal per ha.

Haulm yield

After harvest of stalk from net plot they were threshed and seeds were separated and remaining stalk and straw was recorded as haulm yield (kg/plot) and finally expressed as quintal per ha.

3.8 MICROBIOLOGICAL OBSERVATIONS

3.8.1 Estimation of phyllosphere and rhizosphere *Methylobacterium* population

One gram of representative leaf sample from each treatment was grounded using a pestle and mortar, serially diluted upto 10^6 dilutions and one ml of the aliquot from 10^6 was transferred to the sterile petri dishes. The AMS medium was sterilized and poured into petri dishes. The plates were incubated at $35 \pm 2^\circ\text{C}$ temperature for one week and three replications were maintained. The characteristic pink colonies growing over the medium were counted and expressed as cfu g^{-1} fresh weight of leaf tissue. Similarly, population counts of methylotrrophs from rhizosphere soil was taken and expressed as cfu g^{-1} dry weight of soil.

3.8.2 Enumeration of free Living Nitrogen Fixer (*Azotobacter*) and Phosphate Solubilizers in the Rhizosphere

Azotobacter and phosphate solubilizers

The enumeration of *Azotobacter* and phosphate solubilizers in the fresh soil samples was carried out by following the standard serial dilution plate count technique using Norris N-free agar for free living Nitrogen fixers (Norris, 1959) and Pikovskaya's agar for phosphate solubilizers (Pikovskaya, 1948).

Ten grams of freshly collected soil samples was transferred to 90 ml sterile water blank and shaken for five minutes. Ten ml of this suspension was transferred to a fresh 90 ml sterile water blank and subsequent dilutions upto 10^6 were prepared in the same manner. One ml of suspension from each of the appropriate dilutions was transferred aseptically to sterile petri plates and suitable molten agar medium cooled and poured to the petri plates. The plates were gently rotated in clockwise and anticlockwise directions for uniform distribution of soil suspension. The medium was allowed to solidify and plates were incubated in inverted position at 30°C in an incubator for 3 to 7 days and number of colony forming units was recorded. The population counts were expressed per gram oven dry soil.

3.8.3 Chemical analysis

Enzymes

Dehydrogenase activity

Dehydrogenase in the soil samples was determined by following the procedure as described by Casida *et al.* (1964). Ten grams of soil and 0.2 g CaCO_3 were thoroughly mixed and dispensed in test tubes. To each tube was added one ml of 3 per cent aqueous solution of 2, 3, 5-tiphenyl tetrazolium chloride (TTC), one ml of one per cent glucose solution and eight ml of distilled water which was sufficient to leave a thin film of water above the soil layer. The tubes were stoppered with rubber cork and incubated at 30°C for 24 hrs. At the end of incubation, the contents of the tube were rinsed down into a small beaker and slurry was made by adding 10 ml methanol. The slurry was filtered through watman No. 50 paper. Repeated rinsing of soil with one ml methanol was continued till the filtrate ran free of red colour. The filtrate was pooled and makeup to 50 ml with methanol in a volumetric flask. The intensity of red colour was measured at 485 nm against a methanol blank using Graphicord Shimadzu UV/Visible Spectrophotometer (Model V-240). The concentration of formazan in soil samples were determined by reference to a standard curve prepared by using graded concentration of formazan. The results were expressed as μg of triphenyl formazan (TPF) formed g^{-1} soil per day.

Urease activity

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

Ten grams each of freshly collected soil samples were placed in 100 ml capacity Erlenmeyer flasks to which one ml toluene was added and allowed to stand for 15 min to permit complete penetration into soil. Each of these flask were added with 10 ml of phosphate

buffer (pH 6.7) and 10 ml of 10 per cent urea solution. For control flasks, urea solution was replaced by equal quantity of distilled water. The contents of the flask were well shaken for five minutes and incubated at 30°C for 24 hrs. After incubation, the contents of the flasks were filtered through Watman No. 42 filter paper. The remaining soil in the flask was added with 15 ml of 1 N KCl solution shaken for five minutes and filtered. The volume of the total filtrate was made up to 100 ml in the volumetric flask using distilled water.

The amount of ammonia present in the filtrate was determined by nesslerization. One ml filtrate of each sample was transferred to 20 ml volumetric flask to which 100 ml of 10 per cent sodium tartarate solution and 0.5 ml of Nessler's reagent were added. The volume was made up to 20 ml with distilled water.

The yellow colour developed after 30 min was measured at 410 nm using Graphicon Shimadzu UV/visible spectrophotometer (model UV 240) against the reagent blank.

The results obtained were expressed as µg of ammonia liberated per gram soil (oven dry basis) per day with reference to a standard curve outlined by using graded concentrations (0-100 µg ml⁻¹) of (NH₄)₂SO₄ solution and developing the colour by nesslerization.

Phosphatase activity

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

One gram of soil sample was placed in 50 ml Erlenmeyer flask to which 0.2 ml toluene followed by four ml of modified universal buffer (pH 7.5) was added. One ml of P-nitro phenol phosphate solution made in modified universal buffer was added to the flasks and contents of the flasks were mixed by swirling for 2 min. The flasks were stoppered and incubated at 37°C for one h. After incubation, one ml of 0.5 M CaCl₂ and four ml of 0.5 M NaOH were added to the flask, swirled and filtered through Watman No. 42 filter paper. The intensity of yellow colour developed was measured at 420 nm against the reagent blank using Graphicon Shimadzu UV-visible, Spectrophotometer (model UV-240).

Controls were maintained for each soil sample and were analyzed by following the same procedure described above except that the paranitrophenol phosphate solution was added after the addition of 0.5 M CaCl₂ and 0.5 M NaOH and just before filtration. The phosphatase activity in the soil samples was expressed as µg paranitrophenol formed per gram soil per hour with reference to the standard curve prepared by using graded concentrations of P-nitro phenol phosphate.

3.8.4 Estimation of chlorophyll content in leaf

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 min. At the end of the incubation period, decanted the supernatant and discarded the leaf tissue, made up the volume to 10 ml with DMSO. Read the absorbance of extract at 645 and 663 nm using DMSO as blank.

Calculations:

$$\text{Chlorophyll 'a'} = \frac{12.7 (A_{663}) - 2.69 A_{645} \times V}{1000 \times W \times a} \text{ (mg/g)}$$

$$\text{Chlorophyll 'b'} = \frac{22.9 (A_{645}) - 4.68 A_{663} \times V}{1000 \times W \times a} \text{ (mg/g fresh weight)}$$

$$\text{Total chlorophyll} = \frac{20.2 (A_{645}) - 8.02 A_{663} \times V}{1000 \times W \times a} \text{ (mg/g fresh weight)}$$

Where,

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

V = Final volume of chlorophyll extract (ml)

W = Fresh weight of sample (g)

a = Path length of light (1 cm)

3.8.5 Chemical analysis of plant sample

N uptake by plants

The plant samples collected at 30, 60 and at harvesting were dried and ground in a Wiley mill. The powdered samples were used for the estimation of nitrogen by following the microkjeldhal method (Jackson, 1973). The analysis was done using 500 mg of oven dried finely ground sample (passed through 100 mesh sieve). The samples were digested with 10 ml concentrated sulphuric acid in the presence of 2000 mg catalyst mixture containing potassium sulphate, copper sulphate and selenium in the ratio 100:10:1. The sample was digested at 100 °C overnight and again at 400 °C for 90 min or till the sample become clear in the digestion tubes.

The digested samples were distilled in microkjeldhal assembly. The samples were made alkaline by adding 15 ml of 40 per cent NaOH and the ammonia liberated during distillation was collected in 4 per cent boric acid containing mixed indicator and titrated against 0.05 N H₂SO₄. The percentage of nitrogen was calculated by recording the volume of acid run down. The N uptake was calculated by multiplying the N percentage with the corresponding dry matter yield.

Phosphorus uptake by plant

Five ml of aliquot of the plant digest was transferred into a 50 ml volumetric flask. Ten ml of Vanadomolybdate reagent was added to samples and also to each standards, mixed thoroughly and the volume made upto 50 ml with distilled water. After 30 min of colour development, the intensity of yellow colour was read in a spectrophotometer at 470 nm. The calibration curve (standard graph) of 'P' standard was drawn by plotting the P-absorbance against 'P' concentration. The 'P' content in plant digest sample was determined by referring to the standard curve. The 'P' uptake was calculated by multiplying 'P' percentage with the corresponding dry matter yield.

Potassium uptake

The potassium in the plant samples was estimated by atomizing the diluted plant extract in the flame photometer as described by Jackson (1967).

A known quantity of the aliquot (or) plant digest was transferred to a 50 ml volumetric flask and the volume made upto with distilled water. The flame photometer reading was adjusted to zero with 0 ppm K standard solution and to 100 with 5 ppm K standard solution. The diluted solutions of the plant digest were fed and the flame photometer readings recorded. The standard curve was drawn by plotting flame photometer readings. On Y-axis and concentration of K on X-axis. The concentration of K in diluted plant digested sample was arrived at by referring to the standard curve.

$$\text{Nutrient uptake (kg/ha)} = \frac{\text{Per cent nutrient concentration}}{100} \times \text{biomass (kg/ha)}$$

The data was subjected to Randomized Completely Block Design (RCBD) and Completely Randomized Design (CRD) analysis as described by (Panse and Sukhatme, 1967). The level of significance used in 'F' and 't' test was P=0.05 for field experiment and P=0.01 for lab studies. Critical difference values were calculated whenever the 'F' test values are significant.

4. EXPERIMENTAL RESULTS

A field experiment was conducted in black soil during *rabi* season 2007-08 to evaluate the effect of combined inoculation of *Methylobacterium* and *B. japonicum* (strain *B. japonicum*) on growth, yield, nutrient uptake of soybean and also population dynamics of methylo-trophs and other beneficial rhizosphere microorganisms and soil enzyme activities. The results obtained on these aspects are presented here in.

4.1 GROWTH PARAMETERS

4.1.1 Plant height

The data presented in the Table 3 revealed that the combined inoculation of *Methylobacterium* and *B. japonicum* and foliar spray with *Methylobacterium* isolates significantly increased the plant height both at 30 and 60 DAS.

At 30 days after sowing (DAS) seed inoculation with ML55 and *B. japonicum* with foliar spray (T7) showed the maximum plant height (28.97cm), which was significantly, superior over control T5 (24.67cm) and seed inoculation with ML2 + *B. japonicum* and seed inoculation with ML2 + *B. japonicum* with FS of ML2 (T₆). However it was on par with all other treatments.

At 60 DAS, T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed significantly higher plant height (54.25cm), over seed inoculation with ML₂ and *B. japonicum* and seed inoculation with ML₆₆ + *B. japonicum* and remaining seed inoculation with foliar spray treatments and also with control (T5). At 60 DAS, there is no significant difference between treatments with inoculation of methylo-trophs as compared to control.

4.1.2 Number of leaves

The results on the number of leaves as influenced by different microbial inoculation and foliar spray treatments by *Methylobacterium* and *B.japonicum* are depicted in Table 3.

At 30 DAS T9 (seed inoculation + *B. japonicum* and FS with reference strain) showed more number of leaves 25.33, which was significantly superior over Control T5 (16.66). However all other treatments receiving seed inoculation and seed inoculation along with foliar spray treatments were on par with T9.

A significant difference was observed among the treatments receiving seed inoculation and foliar spray at 60DAS. Treatment T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed higher number of leaves (43.40), which was significantly superior over control (36.60) and other seed inoculation treatments. However T9 was on par with treatments receiving both seed inoculation and foliar spray and also seed inoculation with dual culture T4 (reference strain + *B. japonicum*) and T3 (ML66 + *B. japonicum*) respectively. Whereas control T5 was on par with T1 and T2.

4.1.3 Number of branches

No significant differences were observed among the treatments receiving both seed inoculation with dual culture and foliar spray with *Methylobacterium* isolates on branches of soybean at 30 DAS (Table 3).

At 60 DAS T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed maximum number of branches (12.53/plant), which was significantly superior over T2, 8.87/plant (seed inoculation with ML2 + *B. japonicum*). However T2 was on par with control (10.80/plant). Whereas treatments receiving dual culture and foliar spray were on par with the treatments receiving only seed inoculation with *B. japonicum* and *Methylobacterium* T2 (ML55 + *B. japonicum*), T3 (ML66 + *B. japonicum*) and T4 (reference strain + *B. japonicum*) respectively.

4.1.4 Nodule Number

Variations in nodule number at 45 and 60 DAS as consequent of different microbial inoculation and their interaction are presented in Table 4.

Table 3. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on plant height, number of leaves and branches of soybean at two different crop growth stages

Treatments	Plant height (cm)		Number of leaves/plant		Number of branches/plant	
	30 DAS	60 DAS	30 DAS	60 DAS	30 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	26.57 ^{abc}	36.83 ^c	22.17 ^a	37.53 ^c	5.66	8.77 ^b
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	25.90 ^a	47.33 ^{ab}	22.97 ^a	37.53 ^c	6.20	11.87 ^a
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	27.13 ^{abc}	44.30 ^{bc}	22.80 ^a	38.87 ^{bc}	5.40	11.47 ^a
T ₄ – SI with reference strain and <i>B. japonicum</i>	27.20 ^{abc}	54.25 ^{ab}	23.36 ^a	39.67 ^{bc}	5.93	12.27 ^a
T ₅ – SI with <i>B. japonicum</i> (Control)	24.67 ^c	46.31 ^{ab}	16.66 ^b	36.60 ^c	5.53	10.80 ^{ab}
T ₆ – T ₁ + FS with ML ₂	25.70 ^{bc}	45.46 ^{abc}	22.57 ^a	40.67 ^{abc}	5.60	11.37 ^a
T ₇ – T ₂ + FS with ML ₅₅	28.97 ^a	47.72 ^{ab}	24.40 ^a	42.07 ^{ab}	5.53	12.33 ^a
T ₈ – T ₃ + FS with ML ₆₆	28.07 ^{ab}	46.31 ^{ab}	23.37 ^a	43.40 ^a	5.40	12.27 ^a
T ₉ – T ₄ + FS with reference strain	28.80 ^a	48.25 ^a	25.33 ^a	43.20 ^a	5.93	12.53 ^a
S.Em.±	0.87	2.78	1.49	1.25	0.41	0.72
CD at 5%	2.60	8.34	4.47	3.75	NS	2.17

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

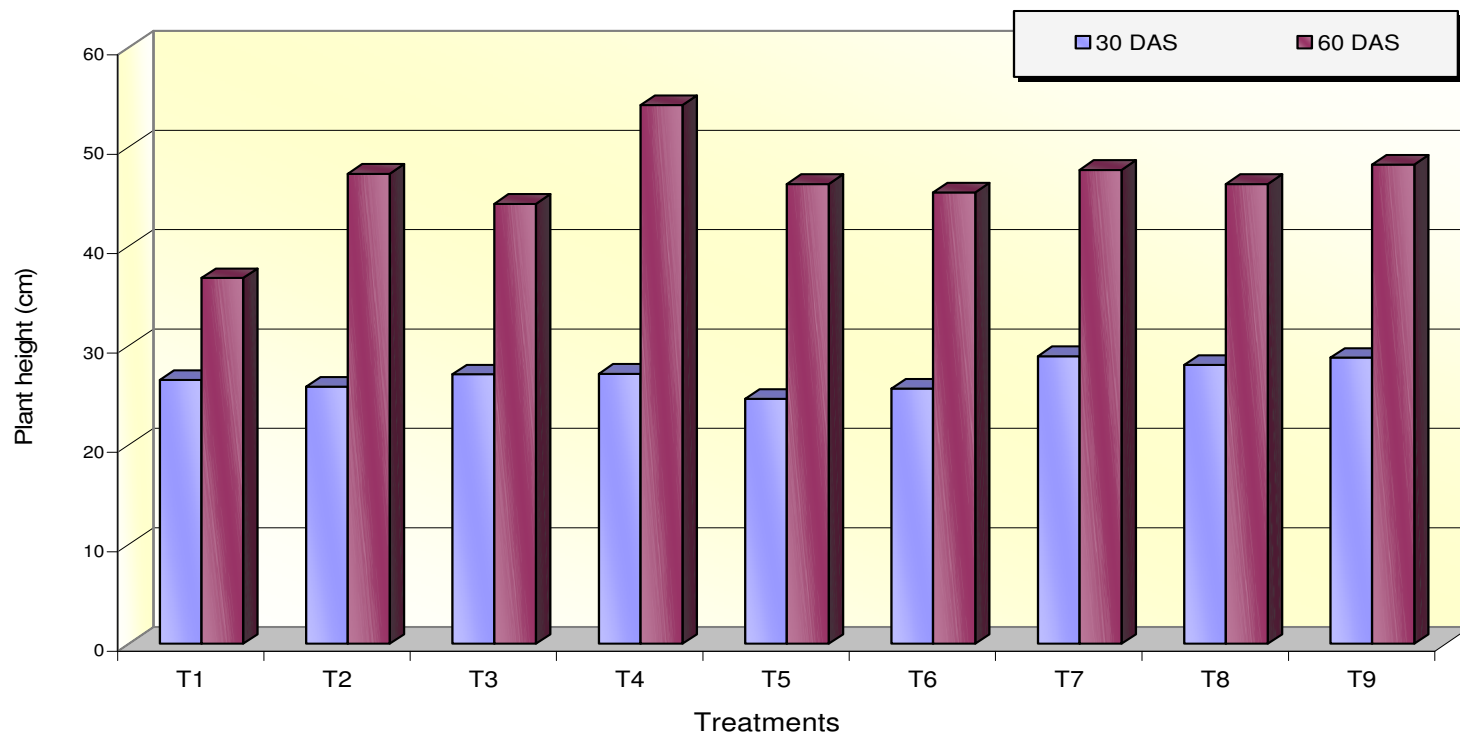


Fig. 2. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on plant height at two different growth stages

Fig. 2. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on plant height at two different growth stages

Table 4. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on nodule count and nodule dry weight of soybean at two different crop growth stages

Treatments	Number of nodules/plant		Nodule dry weight (g/plant)	
	45 DAS	60 DAS	45 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	63.67 ^{bc}	70.33 ^b	0.48 ^{cd}	0.63 ^{bcd}
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	65.67 ^{bc}	70.66 ^b	0.49 ^{cd}	0.47 ^e
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	61.67 ^c	69.00 ^b	0.43 ^d	0.52 ^{de}
T ₄ – SI with reference strain and <i>B. japonicum</i>	67.67 ^{ab}	70.00 ^b	0.53 ^{bc}	0.59 ^{abc}
T ₅ – SI with <i>B. japonicum</i> (Control)	45.67 ^d	49.00 ^c	0.27 ^c	0.31 ^f
T ₆ – T ₁ + FS with ML ₂	63.33 ^{bc}	73.66 ^{ab}	0.60 ^{ab}	0.56 ^{cde}
T ₇ – T ₂ + FS with ML ₅₅	72.33 ^a	75.00 ^{ab}	0.62 ^{ab}	0.68 ^{ab}
T ₈ – T ₃ + FS with ML ₆₆	71.33 ^a	74.33 ^{ab}	0.61 ^{ab}	0.66 ^{ab}
T ₉ – T ₄ + FS with reference strain	72.33 ^a	77.66 ^{ab}	0.64 ^a	0.69 ^a
S.Em.±	1.70	1.97	0.02	0.02
CD at 5%	5.08	5.92	0.08	0.08

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

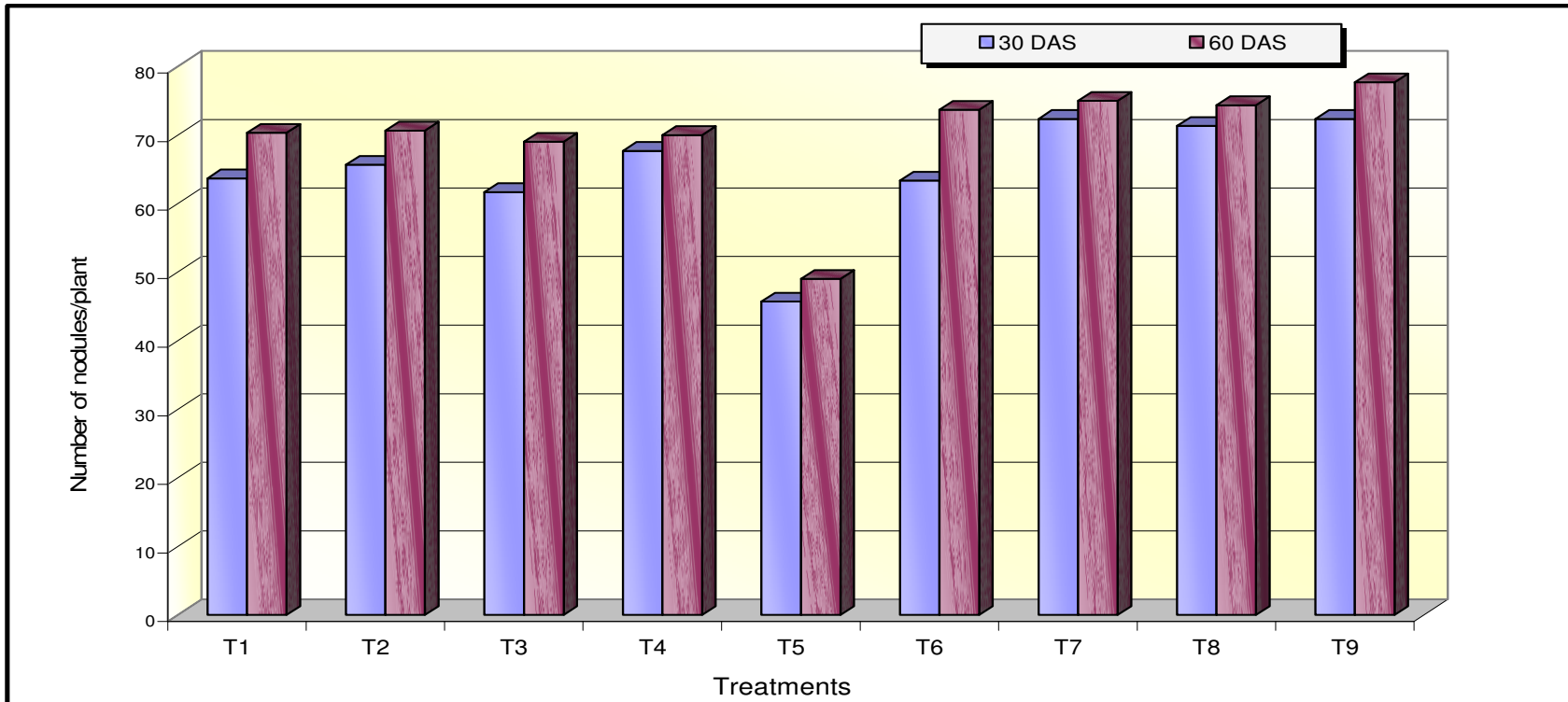


Fig. 3. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on number of nodules/plant at two different growth stages

Fig. 3. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on number of nodules/plant at two different growth stages

At 45 DAS, significantly more number of nodules per plant was recorded in T9 (72.33/plant) which was seed inoculated with reference strain + *B. japonicum* and FS with reference strain followed by T7 and T8 when compared with all other treatments and control (45.67/plant). However, T₁, T₂, T₃ were on par with T₆ (seed inoculation with ML₂ + *B. japonicum* + FS with ML₂) and T₄ was on par with T₇, T₈, T₉ treatments receiving both seed inoculation and foliar spray.

At 60 DAS, the increase in nodule number was observed in all the treatments. T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) recorded more number of nodules (77.66/plant), which was on par with treatments T8, T7 and T6 and statistically superior over control. However treatments receiving seed inoculation with dual culture T1, T2, T3 and T4 were on par with the treatments receiving both seed inoculation and foliar spray.

4.1.5 Nodule dry weight

Significant variations in the nodule dry weight was observed due to seed inoculation with *Methylobacterium* isolates and *B. japonicum* strain *B. japonicum* and foliar spray with *Methylobacterium* isolates. The data on the nodule dry weight is presented in Table 4.

At 45 DAS, significantly higher nodule dry weight of 0.64g/plant was recorded in T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain), which was on par with all the treatments receiving seed inoculation and foliar spray. However significantly over control (0.27 g/plant) and T₁, T₂ and T₃ treatments receiving seed inoculation with dual culture and T₄ (seed inoculation with reference strain + *B. japonicum*) was on par with T₆, T₇, T₈ and T₉ respectively.

At 60 DAS, there was significantly increased nodule dry weight was observed in T9 (seed inoculation with Reference strain + *B. japonicum* and FS with reference strain) 0.69g/plant, which was statistically superior over all seed inoculation treatments with dual culture and on par with treatment T7 and T8 which received both seed inoculation with dual culture and foliar spray and also with control (T₅).

4.2 YIELD PARAMETERS

4.2.1 Shoot dry weight

The results depicted in the Table 5 indicates the information about shoot dry weight of soybean significantly increased due to inoculation of *Methylobacterium* and *B. japonicum* along with foliar spray of *Methylobacterium* isolates.

Initially at 30 DAS, There was no significant ant variation in shoot dry weight of soybean plants.

At 60 DAS, significant variations in the shoot dry weight was observed in T₉ 7.90 g/plant (seed inoculation with reference strain + *B. japonicum* of foliar spray) which was on par with dual inoculation and foliar spray treatments T₇, T₈ and T₄ (seed inoculation with reference strain + *B. japonicum*) and significantly superior over control (6.23 g/plant). Among seed inoculation with dual culture treatments T₄ (reference strain + *B. japonicum*) showed higher shoot dry weight of 6.96g/plant and lowest shoot dry weight of 5.06g/plant was recorded in T₁ (ML₂ + *B. japonicum*). However T₃ was on par with T₂.

4.2.2 Root dry weight

Significant variations among the treatments were observed in root dry weight of soybean plants at all stages of crop growth (Table 5).

At 30DAS T₉ (seed inoculation with reference strain + *B. japonicum* + foliar spray with reference strain) showed higher root dry weight of 1.80 g/plant which was significantly superior over control (0.88 g/plant) and also with seed inoculation with dual culture treatments except T₃ (1.72 g/plant). However T₉ was on par with T₆ (1.63 g/plant). T₇ (1.72 g/plant) and T₈ (1.60 g/plant) which receiving seed inoculation with dual culture and foliar spray with respective isolates.

Table 5. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on shoot and dry matter accumulation of soybean plants at two different crop growth stages

Treatments	Shoot dry weight (g/plant)		Root dry weight (g/plant)		Total dry matter (g/plant)	
	30 DAS	60 DAS	30 DAS	60 DAS	30 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	3.44	5.06 ^f	1.16 ^b	2.26 ^d	4.67 ^b	8.69 ^c
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	3.42	6.96 ^d e	1.20 ^b	2.35 ^d	4.68 ^b	8.90 ^c
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	3.35	6.33 ^e	1.60 ^a	2.33 ^d	4.59 ^b	8.49 ^c
T ₄ – SI with reference strain and <i>B. japonicum</i>	3.59	6.56 ^{cd}	1.17 ^b	2.73 ^c	4.72 ^b	10.01 ^b
T ₅ – SI with <i>B. japonicum</i> (Control)	3.22	6.23 ^e	0.88 ^c	1.81 ^e	4.56 ^b	6.89 ^d
T ₆ – T ₁ + FS with ML ₂	3.47	7.22 ^{bc}	1.63 ^a	2.83 ^{bc}	4.99 ^{ab}	10.05 ^b
T ₇ – T ₂ + FS with ML ₅₅	3.83	7.67 ^{ab}	1.72 ^a	3.27 ^a	5.43 ^a	10.63 ^{ab}
T ₈ – T ₃ + FS with ML ₆₆	3.67	7.58 ^{ab}	1.60 ^a	2.90 ^{bc}	5.02 ^{ab}	10.49 ^{ab}
T ₉ – T ₄ + FS with reference strain	3.78	7.90 ^a	1.80 ^a	3.05 ^a	5.35 ^a	10.93 ^a
S.Em.±	0.16	0.19	0.07	0.08	0.19	0.23
CD at 5%	NS	0.58	0.20	0.24	0.57	0.71

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

There was further increase in root dry weight of soybean plants at 60 DAS, T₉ (3.05 g/plant) which was statistically superior over treatments receiving seed inoculation with dual culture and control T₅ (91.81 g/plant) but on par with T₇ (seed inoculation with ML₅₅ + *B. japonicum* and foliar strain with ML₅₅). Among seed inoculation with dual culture T₄ has higher root dry weight (2.73 g/plant). However T₁, T₂ and T₃ are on par with each other and also on par with T₆ and T₈ treatments receiving with *Methylobacterium* + *B. japonicum* and further spray with respective *Methylobacterium* isolates.

4.2.3 Total dry matter accumulation

Significant results were observed in dry matter accumulation at all stages of crop growth due to various inoculation treatments furnished in the Table 5.

At 30 DAS there was statistically maximum dry matter accumulation of 5.43g/plant in T₇ (seed inoculation with ML₅₅ + *B. japonicum* and foliar spray with ML₅) which was on par with T₆, T₈ and T₉, but superior over control 94.56g/plant. Among seed inoculation all treatments are on par with each other. At 60 DAS T₉ showed maximum dry matter accumulation of 10.39 g/plant which was on par with T₇ (seed inoculation with ML₅₅ + *B. japonicum* and FS with ML₅₅) and T₈ (ML₆₆ + *B. japonicum*) and foliar spray with ML₆₆). However T₉ was statistically superior over control (6.89 g/plant). Among seed inoculation with dual culture treatments T₄ recorded maximum dry matter accumulation (10.01g /plant) followed by T₁, T₂ and T₃, which are on par with each other.

4.2.4 Number of pods

Number of pods per plant was significantly increased due to various combined inoculation of *Methylobacterium* and *B.japonicum* and further foliar spray with *Methylobacterium* isolates. The data pertaining to this is presented in Table 6.

Statistically significant results were recorded in treatment T₉ (seed inoculation with reference strain + *B. japonicum* and FS with reference strain), showed more number of pods (63.00), which was significantly superior over control (40.83) and all seed inoculation treatments with dual culture T₁ (ML₂ + *B. japonicum*) and T₂ (ML₅₅ + *B. japonicum*), T₃ and T₄. However T₄ (reference strain + *B. japonicum*) was on par with treatment T₆ receiving seed inoculation with ML₂ + *B. japonicum* and FS with ML₂.

4.2.5 Test weight of 100 seeds

Statistically no significant results were observed on test weight of soybean seeds among the treatments (Table 6).

4.2.6 Grain yield (g/plant)

Data on grain yield as influenced by combined inoculation of *Methylobacterium* and *B. japonicum* are presented in Table 6.

Among the treatments T₉ (seed inoculation with reference strain + *B. japonicum* and foliar spray with reference strain) noticed higher grain yield of 12.00 g/plant which was statistically superior over control (8.00 g/plant) and with T₁ and T₃ which were seed inoculation only with dual culture. However, T₉ was on par with T₆, T₇, T₈ and also with T₂ and T₄.

4.2.7 Grain yield (q/ha)

The data on grain yield of soybean indicated significant differences between the treatments (Table 6). All the treatments receiving dual inoculation showed significant increase in grain yield over control, which received single inoculation with *Bradyrhizobium*.

Among the treatment T₉ (seed inoculation with reference strain + *B. japonicum* and foliar spray with reference strain) produced significantly higher grain yield (17.13 q/ha) over control and T₁, T₂ and T₃ which were dual inoculation with *methylobacterium* and *B. japonicum*. However, all seed inoculation treatments with dual culture were on par with control. Treatment T₇ (seed inoculation with ML₅₅ + *B. japonicum* and FS with ML₅₅) showed 16.28 q/ha which was statistically superior over control and on par with T₆, T₈ and also T₉ and also with T₄ receiving seed inoculation with dual culture.

Table 6. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on number of pods, test weight and grain yield of soybean

Treatments	Number of pods/ plant	Test weight (g/100 seeds)	Grain yield (g/plant)	Grain yield (q/ha)	Straw yield (q/ha)
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	45.06 ^{cd}	10.42	8.33 ^b	13.68 ^{bc}	35.59 ^c
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	46.20 ^c	10.46	10.00 ^{ab}	13.37 ^{bc}	36.62 ^{bc}
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	48.97 ^{bc}	10.10	8.33 ^b	13.38 ^{bc}	35.33 ^c
T ₄ – SI with reference strain and <i>B. japonicum</i>	53.17 ^b	10.67	10.33 ^{ab}	14.38 ^{abc}	36.62 ^{bc}
T ₅ – SI with <i>B. japonicum</i> (Control)	40.83 ^d	10.64	8.00 ^b	12.34 ^c	33.74 ^c
T ₆ – T ₁ + FS with ML ₂	52.00 ^b	10.38	11.00 ^{ab}	14.39 ^{abc}	37.65 ^{bc}
T ₇ – T ₂ + FS with ML ₅₅	58.67 ^a	10.68	11.33 ^{ab}	16.28 ^{ab}	41.14 ^{ab}
T ₈ – T ₃ + FS with ML ₆₆	61.00 ^a	10.02	11.00 ^{ab}	15.01 ^{abc}	37.24 ^{bc}
T ₉ – T ₄ + FS with reference strain	63.00 ^a	10.63	12.00 ^a	17.13 ^a	43.61 ^a
S.Em.±	1.64	0.33	1.05	0.90	1.60
CD at 5%	4.94	NS	3.13	2.70	4.80

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

4.2.8 Straw Yield

The data on straw yield of soybean significantly differed due to seed inoculation and foliar spray treatments with *Methylobacterium* strains (Table 6).

The treatment foliar spray with reference strain in combination with treatment 4 (seed inoculation with reference strain + *B. japonicum*) produced significantly a higher straw yield (43.61 q/ha) and this was on par with T7 (41.14q/ha) (which was seed inoculation with ML55 + *B. japonicum* and FS with ML55 and statistically superior over control and all other treatments receiving seed inoculation with dual culture. However, all treatments receiving seed inoculation were on par with control and also with T₆ and T₈ receiving both seed inoculation and foliar spray.

4.2.9 Chlorophyll content

Chlorophyll content of soybean leaves was significantly differed at 30 and 60 DAS due to various dual inoculation and foliar treatments and were presented in Table 7.

At 30DAS, T9 (1.72mg/g of tissue) showed significantly maximum chlorophyll content compared to rest of the treatments and control (0.73mg/g of tissue). However treatments receiving only seed inoculation were on par with the treatments receiving dual culture and foliar spray. Among the seed inoculation treatments T4 (seed inoculation with reference strain + *B. japonicum*) showed maximum chlorophyll content of 1.11mg/g of tissue, and minimum chlorophyll content (0.90 mg/g of tissue) was recorded in T2 (seed inoculation with ML2 and *B. japonicum*).

At 60 DAS, significant increase in chlorophyll content was recorded in T9 (2.46 mg/g of tissue), which was on par with all treatments receiving dual inoculation and foliar spray and T4 (seed inoculation with reference strain + *B. japonicum*). However significantly superior among all other treatments and control (1.45 mg/g of tissue). Among seed inoculation treatments T4 (seed inoculation with reference Strain + *B. japonicum*) showed significantly maximum chlorophyll content of 2.11 mg/g of tissue followed by T2 and T3. Whereas T1 (seed inoculation with ML2 + *B. japonicum*) showed minimum chlorophyll content of 1.98mg/g of tissue.

4.2.10 Nitrogen uptake

Signification difference in the nitrogen uptake from shoots of soybean was observed at 30 and 60 DAS. The data regarding 'N' uptake is furnished in Table 8.

There was significant difference in nitrogen uptake of soybean at 30 DAS. Highest shoot nitrogen uptake was noticed in T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed (33.81 kg/ha) which was significantly superior over all treatments and control (23.22 kg/ha). Among seed inoculation with *Methylobacterium* and *B. japonicum* treatment T₄ showed 25.81 kg/ha which was on par with other seed inoculation treatments. At 60 DAS, T9 (Seed inoculation with RS + *B. japonicum* and FS with reference strain) showed highest nitrogen uptake (61.50 kg/ha) which was statistically superior over control (47.06 kg/ha), however on par with treatment T₇ and T₈ receiving seed inoculation with dual culture and foliar spray. Among seed inoculation with dual culture T₄ (Reference strain + *B. japonicum*) showed highest N uptake of 52.33 kg/ha which was on par with all seed inoculation treatments and also with control and T₆ (seed inoculation with ML₂ + *B. japonicum* and foliar spray with ML₂).

4.2.11 Phosphorous uptake

The results with respect to uptake of phosphorous as influenced by microbial inoculation of *Methylobacterium* and *B. japonicum* at 30 and 60 DAS are depicted in the Table 8.

There was significant difference in p uptake at Treatment T₉ (seed inoculation with reference strain + *B. japonicum* and foliar spray with reference strain) which showed highest P uptake of 5.63kg/ha, which was on par with T₇ and superior over control (2.60 kg/ha) and all other treatments. Among seed inoculation treatments T₄ (Reference strain + *B. japonicum*) showed highest 'P' uptake (4.39 kg/ha), which was on par with T₆ and T₈. However T₁ was on par with T₂ and T₃.

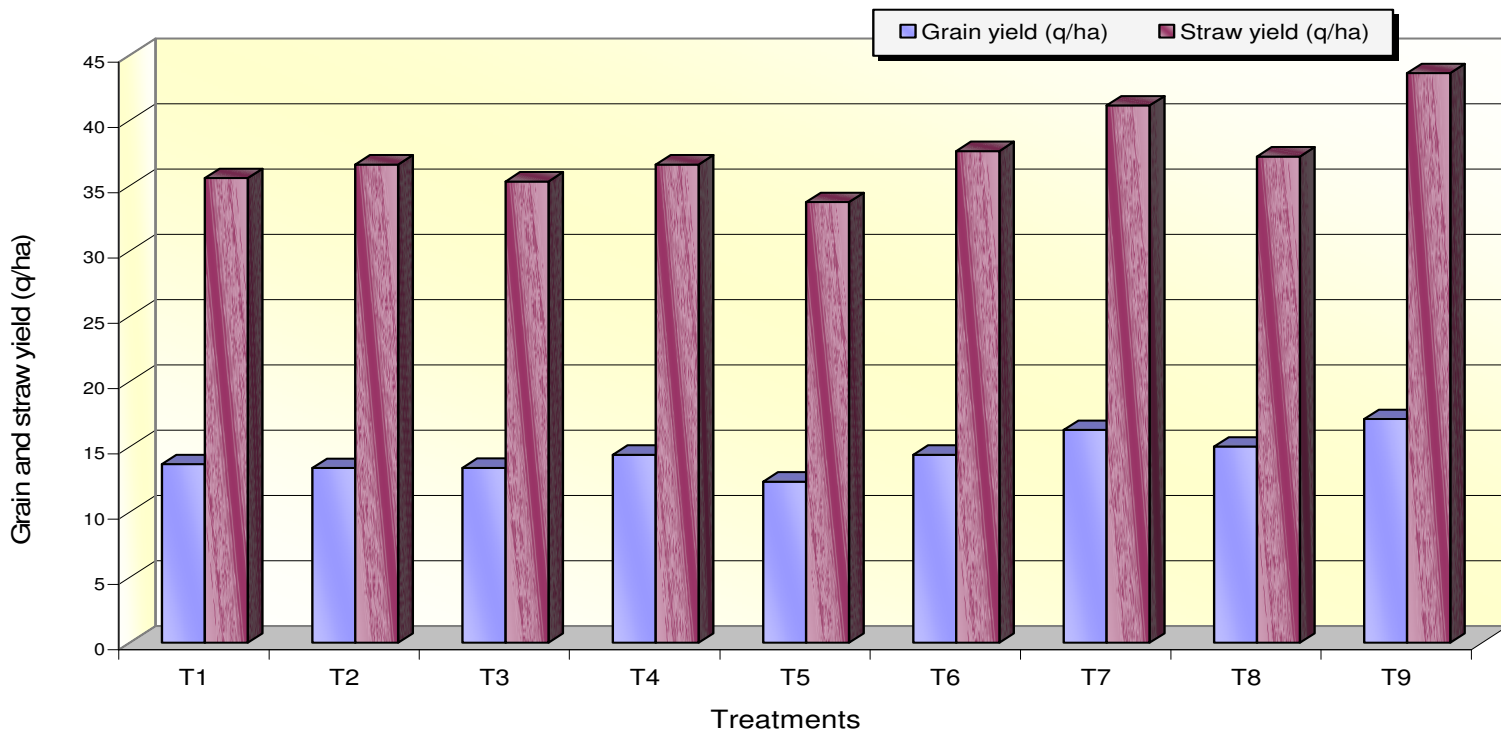


Fig. 4. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on grain and straw yield of soybean plants

Fig. 4. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on grain and straw yield of soybean plants



Plate 4. Seed inoculation with *Bradyrhizobium japonicum* alone

Plate 4. Seed inoculation with *Bradyrhizobium japonicum* alone



Plate 5. Seed inoculation with *Bradyrhizobium japonicum* + *Methylobacterium extorquens* (ME-14718) and foliar spray with ME-14718

Plate 5. Seed inoculation with *Bradyrhizobium japonicum* + *Methylobacterium extorquens* (ME-14718) and foliar spray with ME-14718

Table 7. Chlorophyll content of soybean leaves as influenced by combined inoculation of *Methylobacterium* isolates and *B. japonicum* at two different crop growth stages

Treatments	Chlorophyll content (mg/g of tissue)	
	30 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	0.90 ^{cd}	1.98 ^{bc}
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	1.05 ^{bc}	1.79 ^{cd}
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	1.06 ^{bc}	1.74 ^{cd}
T ₄ – SI with reference strain and <i>B. japonicum</i>	1.11 ^{bc}	2.11 ^{abc}
T ₅ – SI with <i>B. japonicum</i> (Control)	0.73 ^d	1.45 ^d
T ₆ – T ₁ + FS with ML ₂	1.21 ^b	2.26 ^{ab}
T ₇ – T ₂ + FS with ML ₅₅	1.32 ^b	2.28 ^{ab}
T ₈ – T ₃ + FS with ML ₆₆	1.26 ^b	2.27 ^{ab}
T ₉ – T ₄ + FS with reference strain	1.72 ^a	2.46 ^a
S.Em.±	0.08	0.14
CD at 5%	0.26	0.42

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)
Means followed by same letter in the column do not differ significantly by DMRT

Table 8. Nitrogen, phosphorus and potassium uptake of soybean plants as influenced by combined inoculation of *Methylobacterium* isolates and *B. japonicum* at two different crop growth stages

Treatments	N uptake (kg/ha)		P uptake (kg/ha)		K uptake (kg/ha)	
	30 DAS	60 DAS	30 DAS	60 DAS	30 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	23.30 ^d	48.23 ^c	3.42 ^d	4.51 ^d	16.16 ^e	30.50 ^f
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	25.33 ^{cd}	49.83 ^c	3.97 ^{cd}	7.31 ^c	18.52 ^{cd}	44.00 ^{cde}
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	24.19 ^{cd}	50.66 ^c	3.47 ^d	6.20 ^c	19.83 ^{cd}	40.53 ^e
T ₄ – SI with reference strain and <i>B. japonicum</i>	25.81 ^{cd}	52.33 ^{bc}	4.39 ^{bc}	6.93 ^c	18.97 ^{cd}	44.68 ^{cd}
T ₅ – SI with <i>B. japonicum</i> (Control)	23.33 ^d	47.06 ^c	2.60 ^e	4.92 ^d	12.31 ^f	32.63 ^f
T ₆ – T ₁ + FS with ML ₂	26.08 ^c	53.33 ^{bc}	4.56 ^{bc}	8.71 ^b	18.05 ^d	43.34 ^{de}
T ₇ – T ₂ + FS with ML ₅₅	30.24 ^b	61.00 ^a	5.02 ^{ab}	9.98 ^a	21.89 ^b	51.60 ^b
T ₈ – T ₃ + FS with ML ₆₆	31.18 ^b	58.76 ^{ab}	4.81 ^b	9.77 ^{ab}	20.50 ^{bc}	47.37 ^c
T ₉ – T ₄ + FS with reference strain	33.81 ^a	61.50 ^a	5.63 ^a	10.09 ^a	24.87 ^a	54.96 ^a
S.Em.±	0.82	2.12	0.24	0.38	0.62	1.11
CD at 5%	2.48	6.35	0.74	1.16	1.86	3.35

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

At 60 DAS there was statistically significant increase in 'P' uptake in T9 (10.09 kg/ha) compared with control (4.92 kg/ha) and treatments receiving only seed inoculation with dual culture, however among seed inoculation treatment T1 (ML2 + *B. japonicum*) was on par with control and T2, T₃ and T4 were significantly superior over control and on par with each other.

4.2.12 Potassium uptake

A critical perusal of the data present in the Table 8 suggest that there was increase in 'K' uptake by soybean plants as influenced by different inoculation treatments at two growth stages.

From the results it could be seen that there was highest potassium uptake in treatment T₉ (24.87 kg/ha) which was seed inoculation with reference strain + *B. japonicum* and seed inoculation with reference strain, which was significantly superior over seed inoculation treatments and control (12.31 kg/ha). However among seed inoculation treatments highest 'K' uptake of 18.7 kg/ha was seen in T₄ (reference strain + *B. japonicum*) and lowest 'K' uptake (16.16 kg/ha) was seen in T₁ (ML₂ + *B. japonicum*), whereas T₂ and T₃ were on par with T₄.

At 60 DAS, 'K' uptake was superior in treatment T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed 54.96 kg/ha which was significantly higher than control (32.63 kg/ha) and all other treatments followed by T₇ (51.60 kg/ha). However T₁ was on par with control, T₆ was on par with T₂ and T₄ respectively.

4.3 DYNAMICS OF METHYLOBACTERIUM POPULATION IN PHYLLOSPHERE

The data pertaining to the foliar *Methylobacterium* population is presented in Table 9.

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

At 30 DAS T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) recorded maximum population of (35.00×10^3 cfu g⁻¹ fresh weight of leaf tissue) over rest of the treatments and significantly superior over control (16.33×10^3) and on par with all seed inoculation + foliar treatments. Among seed inoculation with dual culture T4 (reference strain + *B. japonicum*) recorded maximum population (30×10^3) which was on par with T2 (29×10^3) and T₃ (28×10^3).

At 60 DAS, there was significant increase in *Methylobacterium* population in T9 (108.88×10^3 cfu g⁻¹ fresh weight of leaf tissue), which was significantly superior over control (87.33×10^3) and T₃. However T9 was on par with T7 (seed inoculation with ML55 + *B. japonicum* and FS with ML55), T8, T6 and also with T₁, T₂ and T₄ which were seed inoculation treatments with dual culture.

4.4 DYNAMICS OF METHYLOBACTERIUM POPULATION IN RHIZOSPHERE

The data regarding to the rhizosphere *Methylobacterium* population is furnished in Table 9.

At 30 DAS, the maximum *Methylobacterium* population in rhizosphere was observed in T9 (19.33×10^6 cfu g⁻¹ dry weight of soil), which was statistically superior over control (13.00×10^6) and on par with all the treatments receiving dual inoculation and foliar spray except T1 (15.33×10^6 cfu g⁻¹ dry weight of soil), which was on par with control.

At 60 DAS, maximum population of *Methylobacterium* in rhizosphere soil was observed in treatment T9 (26.66×10^6 cfu g⁻¹ dry weight of soil) which was superior over control (19.00×10^6). However T9 was on par with T₆ (23.00×10^6), T7 (26.33×10^6), T8 (25.33×10^6) and T₄. But statistically superior over T1, T2 and T3, however, T₁, T₂ and T₃ which were on par with each other.

Table 9. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on PPFM's in phyllosphere and rhizosphere of soybean at two different crop growth stages

Treatments	Phyllosphere (10 ³ cfu g ⁻¹ weight of leaf tissue)		Rhizosphere (10 ⁶ cfu g ⁻¹ dry weight of soil)	
	30 DAS	60 DAS	30 DAS	60 DAS
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	25.66 (7.40) ^d	97.00 (7.98) ^{abc}	15.33 (4.18) ^{bc}	21.33 (4.32) ^{bc}
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	29.00 (7.47) ^{bc}	95.66 (7.98) ^{abc}	16.33 (4.21) ^{ab}	20.66 (4.31) ^{bc}
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	28.00 (7.44) ^{cd}	95.33 (7.97) ^{bc}	15.66 (4.19) ^{abc}	19.66 (4.29) ^{bc}
T ₄ – SI with reference strain and <i>B. japonicum</i>	30.00 (7.48) ^{bc}	100.00 (8.00) ^{ab}	17.33 (4.23) ^{ab}	23.00 (4.36) ^{ab}
T ₅ – SI with <i>B. japonicum</i> (Control)	16.33 (7.21) ^e	87.33 (7.94) ^{cd}	13.00 (4.11) ^c	19.00 (4.27) ^c
T ₆ – T ₁ + FS with ML ₂	30.00 (7.48) ^{bc}	99.66 (7.99) ^{abc}	17.00 (4.22) ^{ab}	23.00 (4.35) ^{abc}
T ₇ – T ₂ + FS with ML ₅₅	33.66 (7.52) ^{ab}	106.00 (8.02) ^{ab}	19.00 (4.27) ^{ab}	26.33 (4.41) ^a
T ₈ – T ₃ + FS with ML ₆₆	31.66 (7.49) ^{abc}	103.33 (8.01) ^{ab}	17.66 (4.24) ^{ab}	25.33 (4.40) ^a
T ₉ – T ₄ + FS with reference strain	35.00 (7.54) ^a	108.66 (8.03) ^a	19.33 (4.28) ^a	26.66 (4.42) ^a
S.Em.±	0.01	0.01	0.02	0.02
CD at 5%	0.05	0.04	0.08	0.07

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

Figures in parenthesis are log transfer values to which the analysis was done

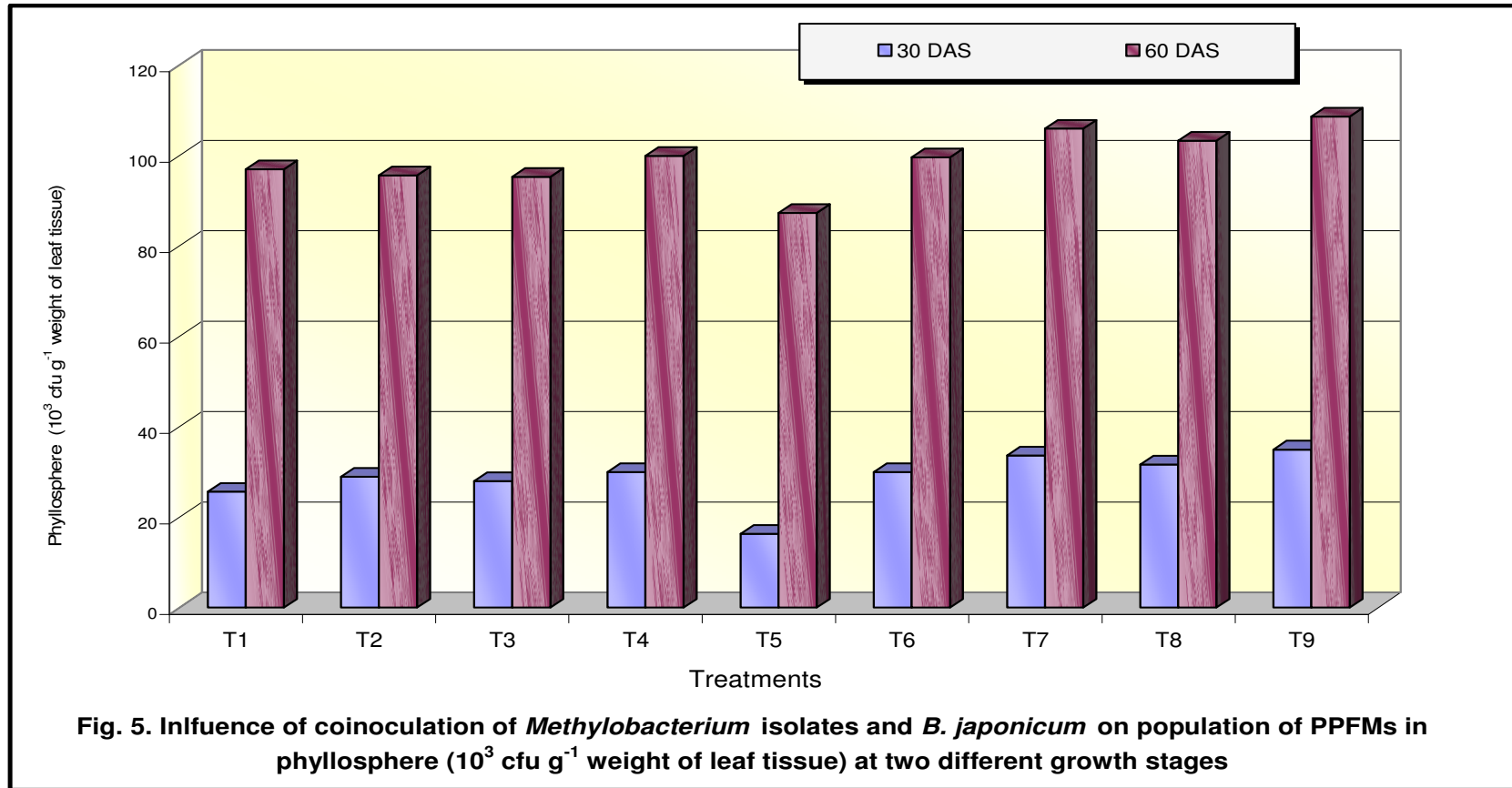


Fig. 5. Influence of coinoculation of *Methylobacterium* isolates and *B. japonicum* on population of PPFMs in phyllosphere (10³ cfu g⁻¹ weight of leaf tissue) at two different growth stages

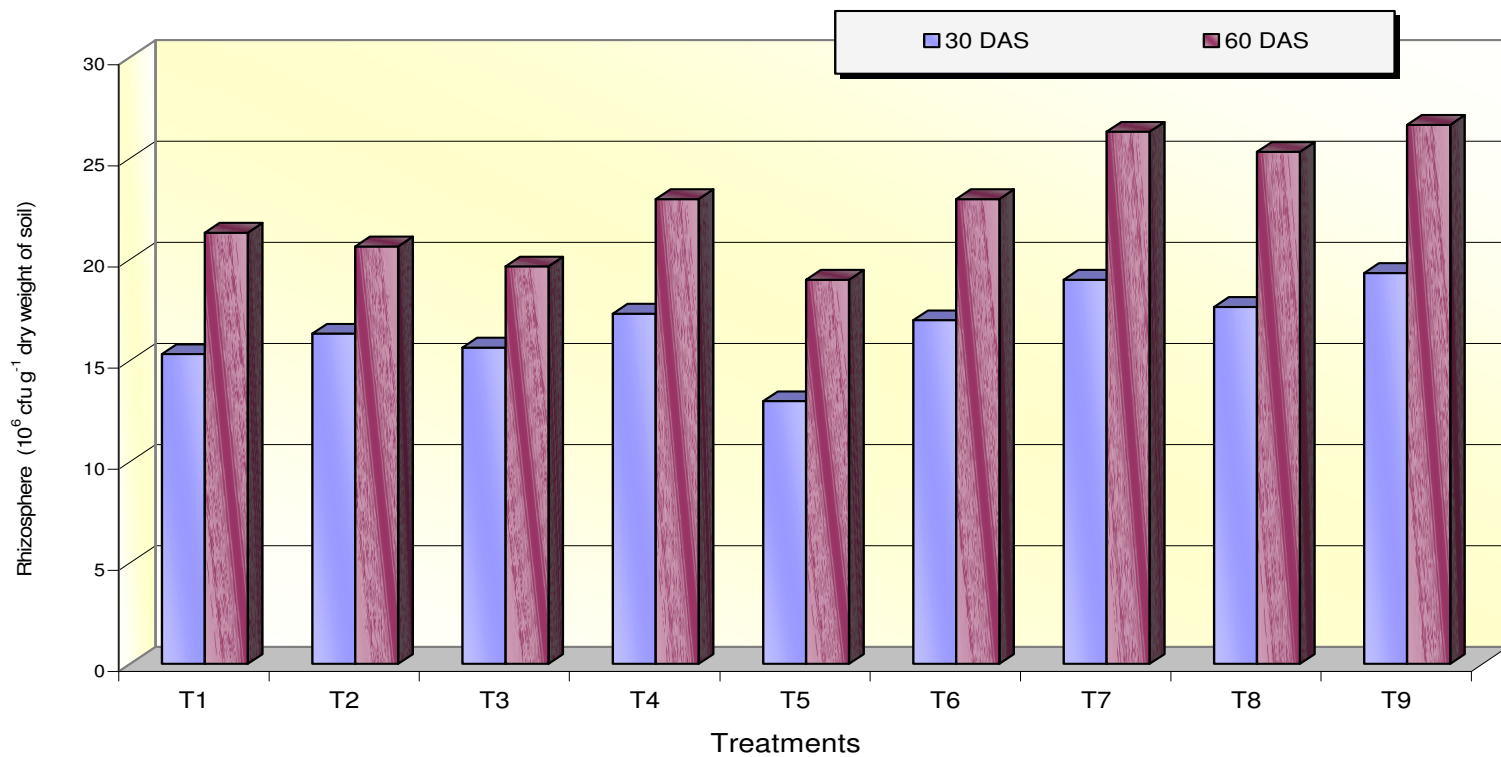


Fig. 6. Influence of coinoculation of *Methylobacterium* isolates and *B. japonicum* on population of PPFMs in rhizosphere soil (10^6 cfu g^{-1} dry weight of soil) at two different growth stages

Fig. 6. Influence of coinoculation of *Methylobacterium* isolates and *B. japonicum* on population of PPFMs in rhizosphere soil (10^6 cfu g^{-1} dry weight of soil) at two different growth stages

4.5 POPULATION OF FREE LIVING NITROGEN FIXERS

4.5.1 *Azotobacter*

Population of *Azotobacter* in rhizosphere soil of soybean significantly differed due to various combined inoculation of *Methylobacterium* and *B. japonicum* and foliar spray of *Methylobacterium* isolates. The data regarding to *Azotobacter* population was depicted in the Table 10.

At 30DAS T9 showed maximum population of 29.33×10^6 cfu g⁻¹ dry weight of soil which was seed inoculation with reference strain and spray with reference strain which was on par with T7 and statistically superior over control (13.00×10^6) and all seed inoculation treatments. Among seed inoculation with dual culture T4 showed maximum population of 18.67×10^6 followed by T1, T2 and T3 which are on par to each other.

At 60 DAS, maximum population (53.33×10^6 cfu g⁻¹ dry weight of soil) was noticed in T9 which was on par with all treatments receiving seed inoculation and foliar spray but significantly superior over all other seed inoculation treatments and control. However, control was on par with T₁.

At harvest statistically no significant results were observed on *Azotobacter* population in rhizosphere soil of soybean.

4.5.2 Phosphate solubilizers

The results on the population of phosphate solubilizing microorganisms of soil as influenced by combined inoculation of *Methylobacterium* and *B.japonicum* are furnished in Table 11.

Significant difference in phosphate solubilizers at 30 DAS was observed in T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain), 7.33×10^3 cfu g⁻¹ dry weight of soil, which was statistically superior over control (3.25×10^3), and on par with T₇. Whereas T₃ was on par with T₁, T₂ and T₄ and also with T₆.

At 60DAS more number of phosphate solubilizers was noticed in T9 (8.67×10^3 cfu g⁻¹ dry weight of soil), which was on par with T7 (8.00×10^3) and statistically superior over control (4.33×10^3) and all other treatments. However T1 receiving only seed inoculation with dual culture was on par with T6 and T8 treatments receiving both seed inoculation and foliar spray.

No significant differences were noticed among the treatments on phosphate solubilizing bacteria at harvest.

4.5.4 Dehydrogenase Activity

The results on dehydrogenase activity in rhizosphere soil of soybean as influenced by co-inoculation and foliar spray of *Methylobacterium* isolates recorded significant results at all stages of crop growth, are presented in Table 12.

At 30 days after sowing (DAS) the dehydrogenase activity of soil was highest in treatment T9 (seed inoculation with reference strain + *B. japonicum* and foliar spray of reference strain) showed (26.78 µg) significantly superior over all seed inoculation treatments and control (11.11 µg) and all other treatments. However, T3 (seed inoculation with ML66+*B. japonicum*) were on par with control and T8 (seed inoculation with ML55 + *B. japonicum* and FS with ML55) was on par with T4 (seed inoculation with reference strain + *B. japonicum*). In general treatments receiving both seed inoculation and foliar spray showed significantly higher dehydrogenase activity over those receiving only seed inoculation.

At 60 DAS, dehydrogenase activity of soil was highest in T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) recorded (27.89µg), which was on par with T8 (seed inoculation with ML66 + *B. japonicum* and FS with ML66) and superior over control (12.09µg) and also with other seed inoculation treatments. However T4 (seed inoculation with reference strain + *B. japonicum*) was on par with T6 (seed inoculation with ML2 and *B. japonicum* + FS with ML2). Among the seed inoculation treatments T1 (seed inoculation with ML2 + *B. japonicum*), T2 (seed inoculation with ML55 + *B. japonicum*) and T₃ (seed inoculation with ML₆₆ + *B. japonicum*) were on par with each other.

Table 10. Population of free living nitrogen fixer in rhizosphere soil of soybean as influenced by combined inoculation of *Methylobacterium* isolates and *B. japonicum* at different crop growth stages

Treatments	Azotobacter (10^6 cfu g ⁻¹ dry wt. of soil)		
	30 DAS	60 DAS	At harvest
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	17.00 (7.23) ^{cd}	32.67 (7.51) ^{cd}	14.00 (7.14)
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	16.00 (7.20) ^{cd}	34.00 (7.53) ^c	12.33 (7.05)
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	16.00 (7.20) ^{cd}	30.00 (7.48) ^c	9.00 (6.95)
T ₄ – SI with reference strain and <i>B. japonicum</i>	18.67 (7.27) ^c	35.67 (7.55) ^{bc}	10.33 (6.99)
T ₅ – SI with <i>B. japonicum</i> (Control)	13.00 (7.12) ^d	26.00 (7.41) ^d	8.00 (6.8)
T ₆ – T ₁ + FS with ML ₂	18.67 (7.27) ^c	51.33 (7.64) ^{ab}	12.00 (7.07)
T ₇ – T ₂ + FS with ML ₅₅	25.33 (7.40) ^a	51.00 (7.71) ^a	16.33 (7.21)
T ₈ – T ₃ + FS with ML ₆₆	20.00 (7.30) ^{bc}	49.00 (7.70) ^a	15.33 (7.18)
T ₉ – T ₄ + FS with reference strain	29.33 (7.46) ^a	53.33 (7.73) ^a	21.00 (7.32)
S.Em.±	0.04	0.03	0.07
CD at 1%	0.16	0.14	NS

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

Figures in parenthesis are log transfer values to which the analysis was done

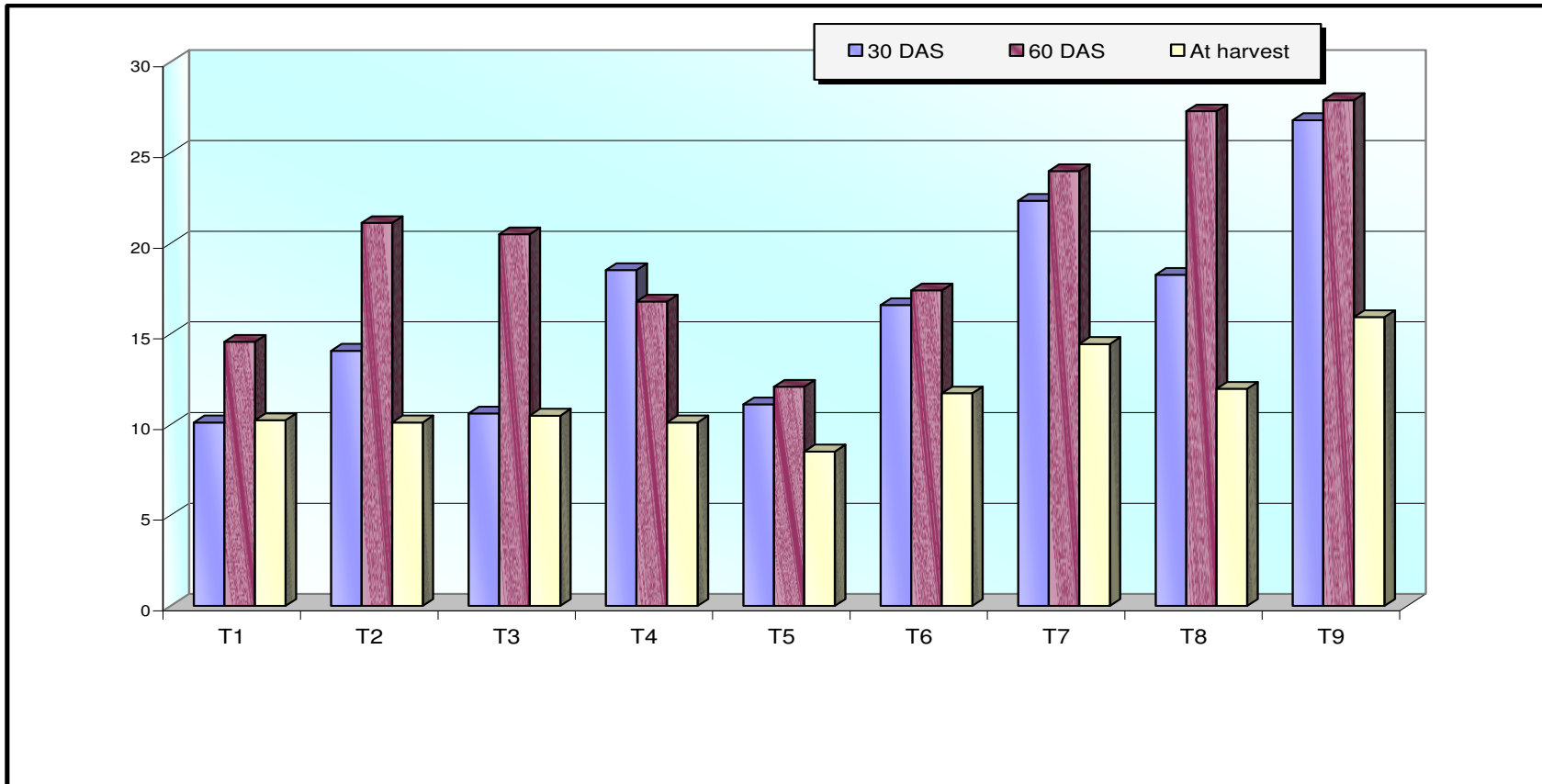


Table 11. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on phosphate solubilizing bacteria (PSB) in rhizosphere soil of soybean at different crop growth stages

Treatments	Phosphate solubilizing bacteria (PSB) (10^3 cfu g ⁻¹ dry wt. of soil)		
	30 DAS	60 DAS	At harvest
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	5.00 (3.69) ^{bcd}	6.33 (3.80) ^c	3.00 (3.46)
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	4.33 (3.63) ^{bcd}	5.00 (3.70) ^d	2.33 (3.36)
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	4.00 (3.59) ^{cd}	4.67 (3.67) ^{de}	2.67 (3.42)
T ₄ – SI with reference strain and <i>B. japonicum</i>	4.67 (3.66) ^{bcd}	5.33 (3.73) ^d	3.00 (3.46)
T ₅ – SI with <i>B. japonicum</i> (Control)	3.33 (3.52) ^d	4.33 (3.63) ^e	3.33 (3.49)
T ₆ – T ₁ + FS with ML ₂	5.00 (3.69) ^{bcd}	7.00 (3.85) ^{bc}	2.67 (3.42)
T ₇ – T ₂ + FS with ML ₅₅	6.33 (3.80) ^{ab}	8.00 (3.90) ^{ab}	4.33 (3.62)
T ₈ – T ₃ + FS with ML ₆₆	5.67 (3.75) ^{abc}	7.33 (3.86) ^{bc}	4.00 (3.59)
T ₉ – T ₄ + FS with reference strain	7.33 (3.86) ^a	8.67 (3.94) ^a	5.00 (3.69)
S.Em.±	0.05	0.02	0.08
CD at 5%	0.22	0.09	NS

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)

Means followed by same letter in the column do not differ significantly by DMRT

Figures in parenthesis are log transfer values to which the analysis was done

Table 12. Effect of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on dehydrogenase activity in rhizosphere of soybean soil at different crop growth stages

Treatments	Dehydrogenase activity ($\mu\text{g TPF g}^{-1} \text{soil d}^{-1}$)		
	30 DAS	60 DAS	At harvest
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	10.12 ^f	14.56 ^c	10.24 ^c
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	14.07 ^e	21.11 ^c	10.12 ^c
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	10.61 ^f	20.49 ^c	10.49 ^c
T ₄ – SI with reference strain and <i>B. japonicum</i>	18.51 ^c	16.78 ^d	10.12 ^c
T ₅ – SI with <i>B. japonicum</i> (Control)	11.11 ^f	12.09 ^f	8.51 ^d
T ₆ – T ₁ + FS with ML ₂	16.59 ^d	17.40 ^d	11.72 ^{bc}
T ₇ – T ₂ + FS with ML ₅₅	22.34 ^b	23.97 ^b	14.44 ^{ab}
T ₈ – T ₃ + FS with ML ₆₆	18.26 ^c	27.27 ^a	11.97 ^{bc}
T ₉ – T ₄ + FS with reference strain	26.78 ^a	27.89 ^a	15.92 ^a
S.Em.±	0.35	0.61	1.10
CD at 5%	1.07	1.85	3.30

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)
Means followed by same letter in the column do not differ significantly by DMRT

At harvest, dehydrogenase activity in soil reduced generally in all treatments, although the extent of reduction was highest in treatment T9 (15.92 μg), which was on par with T7 (seed inoculation with ML55 + *B. japonicum* and FS with ML55) and superior over control (8.51 μg) and rest of the seed inoculation treatments. Among the seed inoculation there was no significant difference among treatments was observed and they were closely on par with each other.

4.5.5 Urease Activity

The data presented in Table 13 indicates the urease activity of soil as influenced by co-inoculation of *Methylobacterium* and *B. japonicum* has significantly differed at all stages of crop growth.

The initial urease activity at 30DAS in the present experiment was highest in T9 (11.30 $\mu\text{g NH}_4\text{-N g}^{-1}\text{ soil}^{-1}$), which was seed inoculation with reference strain + *B. japonicum* and foliar spray with reference strain, significantly superior over control (7.25 μg) and on par with T7 (seed inoculation with ML55 + *B. japonicum* and FS with ML55) and T4 (seed inoculation with reference strain + *B. japonicum*). Among the treatments lowest urease activity was in T6 (seed inoculation with ML2 + *B. japonicum* and FS with ML2), which was on par with the control. However, seed inoculation treatments T1, T2 and T3 were on par with the treatment T8 (seed inoculation with ML66 + *B. japonicum* and FS with ML66).

At 60 DAS, urease activity in soil differed significantly in which treatment T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) showed highest urease activity (27.49 $\mu\text{g NH}_4\text{-N g}^{-1}\text{ solid}^{-1}$) followed by T7 (24.99 μg), which were significantly superior over control (10.23 μg) and all other seed inoculation treatments. Among seed inoculation treatments T4 (Reference + *B. japonicum*) was on par with T8 (seed inoculation with ML66 + *B. japonicum* and FS with ML66) and T₁ (seed inoculation with ML₂ + *B. japonicum* + FS with ML₂).

In general seed inoculation treatments showed lowest urease activity compared to treatments receiving both seed inoculation and foliar spray.

At harvest, dehydrogenase activity in soil reduced generally in all treatments although the extent of reduction was highest in T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) recorded 18.09 which was on par with T7 (seed inoculation with ML55 + *B. japonicum* and FS with ML55) and significantly superior over all seed inoculation treatments. However among seed inoculation treatments T4 recorded. (2.61 μg) followed by T1, T2 and T3, which were on par with each other.

4.5.6 Phosphatase activity

The results on soil phosphatase activity in rhizosphere soil of soybean are depicted in Table 14.

Significant results were observed by combined inoculation of *Methylobacterium* and *B. japonicum* and foliar spray with respective isolates.

The phosphate activity at 30DAS was highest in treatment T9 (seed inoculation with reference strain + *B. japonicum* and FS with reference strain) 8.12 $\mu\text{g pnp g}^{-1}\text{ soil h}^{-1}$ which was statistically superior over all the seed inoculation with dual culture treatments and control (3.08 $\mu\text{g pnp g}^{-1}\text{ soil h}^{-1}$), followed by T7 (6.37 μg). However, T₃ was on par with T₈ and T₁ and T₆ over on par with control.

At 60 DAS significant differences were seen among the treatments. Treatment receiving both seed inoculation and foliar spray (T9) recorded maximum phosphate activity of 11.33 μg , which was significantly superior over all the treatments and on par with T7 (seed inoculation with ML55 + *B. japonicum* and FS with ML55). Minimum phosphatase activity of 8.75 μg was recorded in T5 (seed inoculation with *B. japonicum*), which was on par with T1 and T2 and also with T6 (9.20 μg).

At harvest there was no significant results were observed on phosphatase activity among the treatments.

In general both at 30 and 60 DAS T9 showed significantly higher phosphatase activity.

Table 13. Urease activity in rhizosphere of soybean soil as influenced by combined inoculation of *Methylobacterium* isolates and *B. japonicum* at different crop growth stages

Treatments	Urease activity ($\mu\text{g}/\text{NH}_4\text{-N g}^{-1}$ soil/day)		
	30 DAS	60 DAS	At harvest
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	8.09 ^{bc}	18.21 ^d	10.23 ^d
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	8.44 ^{bc}	14.99 ^{ef}	9.99 ^d
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	8.09 ^{bc}	13.68 ^f	9.28 ^d
T ₄ – SI with reference strain and <i>B. japonicum</i>	10.59 ^a	20.11 ^{cd}	12.61 ^c
T ₅ – SI with <i>B. japonicum</i> (Control)	7.25 ^{cd}	10.23 ^g	7.49 ^e
T ₆ – T ₁ + FS with ML ₂	6.54 ^d	15.94 ^e	13.68 ^b
T ₇ – T ₂ + FS with ML ₅₅	10.59 ^a	24.99 ^b	17.97 ^a
T ₈ – T ₃ + FS with ML ₆₆	8.80 ^b	21.06 ^c	14.28 ^b
T ₉ – T ₄ + FS with reference strain	11.30 ^a	27.49 ^a	18.09 ^a
S.Em.±	0.42	0.64	0.35
CD at 5%	1.26	1.93	1.06

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)
Means followed by same letter in the column do not differ significantly by DMRT

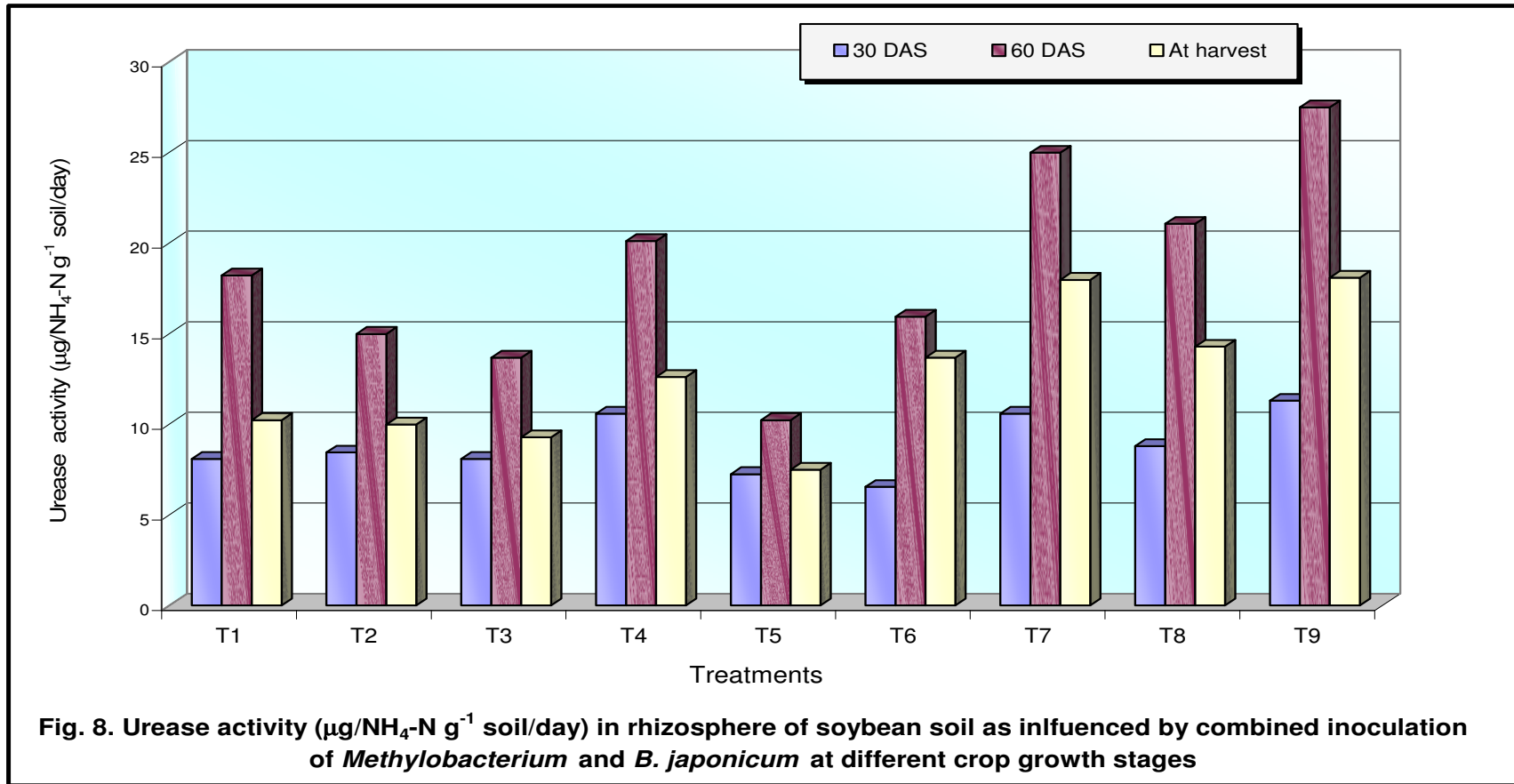


Fig. 8. Urease activity ($\mu\text{g}/\text{NH}_4\text{-N g}^{-1} \text{ soil/day}$) in rhizosphere of soybean soil as influenced by combined inoculation of *Methylobacterium* and *B. japonicum* at different crop growth stages

Table 14. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on phosphatase activity in rhizosphere of soybean soil at different crop growth stages

Treatments	Phosphatase activity ($\mu\text{g } pnp \text{ g}^{-1} \text{ soil h}^{-1}$)		
	30 DAS	60 DAS	At harvest
T ₁ – SI with ML ₂ and <i>B. japonicum</i>	3.20 ^f	9.29 ^{cd}	2.30
T ₂ – SI with ML ₅₅ and <i>B. japonicum</i>	4.62 ^d	10.87 ^c	2.00
T ₃ – SI with ML ₆₆ and <i>B. japonicum</i>	3.95 ^c	9.54 ^{cd}	1.88
T ₄ – SI with reference strain and <i>B. japonicum</i>	5.12 ^e	9.62 ^b	2.45
T ₅ – SI with <i>B. japonicum</i> (Control)	3.08 ^f	8.75 ^d	2.13
T ₆ – T ₁ + FS with ML ₂	3.20 ^f	9.20 ^{cd}	2.20
T ₇ – T ₂ + FS with ML ₅₅	6.37 ^b	11.00 ^{ab}	2.12
T ₈ – T ₃ + FS with ML ₆₆	5.37 ^c	10.87 ^b	2.29
T ₉ – T ₄ + FS with reference strain	8.12 ^a	11.33 ^a	3.04
S.Em.±	0.16	0.25	0.21
CD at 5%	0.48	0.77	NS

SI – Seed inoculation, FS – Foliar spray, Reference strain – *Methylobacterium extorquens* (ME-14718)
Means followed by same letter in the column do not differ significantly by DMRT

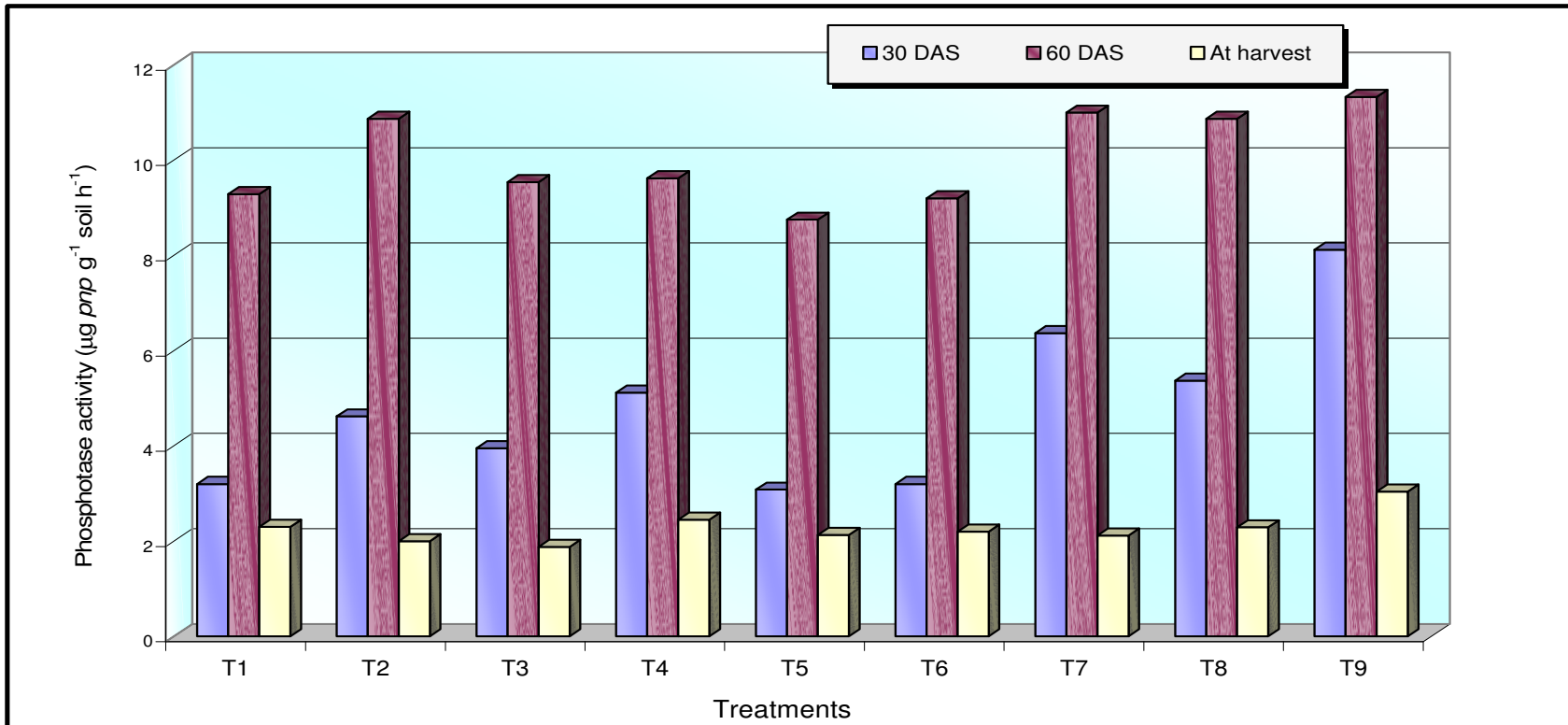


Fig. 9. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on phosphatase activity ($\mu\text{g TPF g}^{-1} \text{ soil d}^{-1}$) in rhizosphere of soil at different crop growth stages

Fig. 9. Influence of combined inoculation of *Methylobacterium* isolates and *B. japonicum* on phosphatase activity ($\mu\text{g TPF g}^{-1} \text{ soil d}^{-1}$) in rhizosphere of soil at different crop growth stages

5. DISCUSSION

Soybean is an important legume crop and a rich source of protein and unsaturated fatty acids. The *Rhizobium* that nodulates soybean is grouped under *B. japonicum* with slow growing polysaccharide and alkali producing characters. *Rhizobium*-legume symbiosis has its own importance in biological nitrogen fixation scenario.

The rhizosphere is a complex system consisting of the soil immediately adjacent to the roots, the root surface with its overlaying slime coat and the endorhizosphere. The microbial activity is influenced by root activities such as exudation of organic substrates such as amino acids, 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 the soil microflora and, thereby, could have major effects on the growth and development of plants (Parvatham *et al.*, 1989). 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 has 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 inoculant, combined inoculation of two or more beneficial and compatible microorganisms has been shown to work better which indicates that in mixed cultures there is a better interaction of the introduced compatible organisms (Alagawadi and Guar, 1988; Poi *et al.*, 1989; Patil *et al.*, 1992; Belimov *et al.*, 1995). Combined inoculations are said to work better than single inoculation based on the principle that greater the diversity and number of inhabitants, the higher the order of interaction and more stable the ecosystem will be (Higa and Wididana, 1991).

Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth promoting regulators like zeatin and related cytokinins and auxins (Dileepkumar and Dube, 1992; Holland and Polacco, 1992, 1994; Holland, 1997). Soybean (*Glycine max*) is a fascinating crop with innumerable possibilities of not only improving agriculture but also supporting industries.

In this context, an attempt was made to study the effect of combined inoculation of *Methylobacterium* isolates and *Bradyrhizobium japonicum* on growth, yield, nutrient uptake in soybean as well as rhizosphere enzyme activities. The results obtained by this present investigations are discussed in this chapter.

5.1 EFFECT OF COMBINED INOCULATION OF *METHYLOBACTERIUM* AND *Bradyrhizobium japonicum* ON GROWTH AND YIELD OF SOYBEAN

5.1.1 Growth parameters

Seed inoculation of *Methylobacterium* isolates in combination with *Bradyrhizobium japonicum* strain (SB₁₂₀) and further foliar spraying with *Methylobacterium* isolates had significant influence on different plant growth parameters.

There was significant increase in the plant growth parameters like plant height, leaf number and branches.

Among the different treatments, the plant height, number of leaves and branches of soybean cultivar JS-335 increased significantly with the dual inoculation plus foliar spray when compared with only dual inoculation with *Methylobacterium* and *B. japonicum*. The maximum plant height was recorded in treatment T₇ receiving both dual inoculation plus foliar spray which was significantly superior over control by 17.43 per cent. However, this was on par with all other treatments receiving both combined inoculation with dual culture and foliar spray. Similarly, higher number of leaves and branches were recorded in T₉ followed by T₇. In all the parameters, the least height, number of leaves and also branches were recorded in treatment T₅ which is seed inoculated only with *B. japonicum* (*B. japonicum*). Basile *et al.* (1969) first demonstrated the growth enhancing effects of PPFMs on plants. In a tissue

culture system, they demonstrated that PPFMs produced a diffusible substance that stimulated the growth of *Scapania nemarosa*, a liverwort with which the bacterium is regularly associated. Later, they identified this compound as vitamin B₁₂.

In the present study, the increased plant growth may be due to the increased amounts of cytokinins in plants due to the inoculation with methylotrophs. Similar results were obtained by Holland and Polacco (1994) on soybean which he attributed to the production of vitamin B₁₂ by PPFMs as described by Basle *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 inoculated control, only with *B. japonicum* (*B. japonicum*). 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).

In the present study, increased plant growth may be due to the coordination between auxin and cytokinins, allowing 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.

5.2 YIELD AND YIELD ATTRIBUTES

5.2.1 Nodulation and nodule dry weight

The number of nodules and nodule dry weight were maximum in the treatment T₉ receiving seed inoculation with dual culture plus foliar spray. Nodule number increased by 58.4 per cent when compared with control receiving seed inoculation with *B. japonicum* only. Treatment T₉ was significantly superior over control both at 45 and 60 DAS in nodule number and dry weight of nodules. The increase in the nodule number and nodule dry weight can be attributed to the presence of rhizobia in the legume rhizosphere influencing the legume roots to release growth promoting substances which, in turn might have enhanced the growth of *Methylobacterium in situ* and synergistic effect might have occurred in treatments 9 and 7. The maximum nodule number due to nodulation 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 Gaur, 1988; Balachander and Nagarajan, 1999).

Due to flavonoid compounds selected by the roots of soybean plants helps to release naringenin, genistein and diadzein (iso flavones). So release of these flavonoid molecules by legumes have been found to induce (or) block the transcription of *nod* genes in *rhizobium* and *Bradyrhizobium* and results in increased nodulation as well as nitrogen fixation (Subba Rao, 1999).

5.2.2 Pods and Grain yield

The yield parameters such as number of pods per plant and grain yield were highest in treatment T₉ (seed inoculation with Reference strain + *B. japonicum*) and foliar spray Reference strain showed 54.2 per cent increase in pods over control which was inoculated with *B. japonicum* only. T₉ also showed increased grain yield of about 38 per cent compared with rest of the treatments receiving seed inoculation with dual culture and control receiving seed inoculation only with *Bradyrhizobium japonicum*. However, treatment receiving seed inoculation with ML₅₅ + *B. japonicum* and foliar spray with ML₅₅ showed a 32 per cent higher grain yield compared with control. However, the maximum increase in yield parameters due to dual and combined inoculation of PGPRs as documented (Alagawadi and Gaur, 1988; Patil *et al.*, 1992; Belimov *et al.*, 1995 and Pratiba *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 and foliar spray by methylotrophs which are plant growth promoting foliar (PGPP) bacteria and on rhizosphere by *rhizobium* which is nitrogen fixing bacterium might have resulted in increased plant growth and yield parameters.

5.2.3 Dry matter production

Shoot and root dry weight of soybean plants was significantly higher in treatment T₉ seed inoculation with reference strain and further spray with reference strain followed by T₇ receiving seed inoculation with ML₅₅ + *B. japonicum* and foliar spray with ML₅₅. Both

treatments showed a 17.39% increase in the plant dry weight compared with control. The increase in the plant dry weight due to inoculation of *Rhizobium*, *Azospirillum* and phosphate solubilizers has already been well established (Wani *et al.*, 1978; EL-Demerdash *et al.*, 1992). Similar results of increase in the yield parameters was because of several factors such as release of growth promoting substances like cytokinins and vitamins (Madhaiyan *et al.*, 2004). Similarly 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. Gibberellic acid 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). 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.

These results are similar with the various literature on the beneficial aspects of pink pigmented *Methylobacterium* as 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 (2002) worked on the effect of combined inoculation of PPFMs and *Rhizobium* on groundnut cultivar Co (Gn) 4 and observed that there was significantly increase in plant growth, biomass production and yield parameters of groundnut.

5.2.4 Chlorophyll content

In the present study, there was significant increase in chlorophyll content which was observed in T₉ (seed inoculation with RS + *B. japonicum* and foliar spray with RS) followed by T₇ (seed inoculation with ML₅₅ + *B. japonicum* foliar spray with ML₅₅ which was statistically superior over control T₅ which was seed inoculation only with *Bradyrhizobium japonicum*. The results have shown conformity with the observations made by Madhaiyan *et al.* (2004). They observed higher photosynthetic activity by enhancing the number of stomata, chlorophyll concentration and maleic acid content of crops. Paulraj (2002) have also documented varied levels of chlorophyll content in cardamom, rubber and coffee respectively due to bioinoculation with *methylobacterium*.

5.2.5 Population of PPFM in foliar and rhizosphere

In the present investigation, the foliar spraying of methylotrophs had significantly influenced the foliar and rhizosphere *Methylobacterium* population after 30 DAS. Treatment receiving both spray and inoculation with *Methylobacterium* isolates and *B. japonicum* was noticed (24.42 per cent increase in the population of methylotrophs in foliar when compared with control). Treatment T₉ and T₇ receiving consortia of seed inoculation with dual culture and spray showed statistically higher population (40 per cent increase in rhizosphere population when compared with treatment receiving single inoculation with *Bradyrhizobium japonicum*).

These results are mainly due to the growth hormone production by *Methylobacterium* sp. especially high cytokinin production in apical plant tissues and rhizosphere soil. The foliar spraying PPFMs significantly influenced the *Methylobacterium* population in the foliar of rice crop (Holland, 1997). Similarly Madhaiyan *et al.* (2005) have recorded yield increases in cotton and sugarcane following foliar spray of pink pigmented facultative methylotrophs.

Similar results was noticed by Freyermuth *et al.* (1996), the population of PPFMs ranged from 10⁵ to 10⁹ cfu g⁻¹ fresh tissue and they could not be dismissed as accidental visitors to the plant surface. The fact that their numbers are more in actively growing regions and their numbers are regulated by dilution as the plant tissue on which they are living expand away from the growing tissue are also worth considering.

5.3 FREE LIVING NITROGEN FIXERS

Effect of *Methylobacterium* and *Bradyrhizobium japonicum* on rhizosphere microbial population were seen in rhizosphere of plants compared to control.

Azotobacter population was significantly superior in the treatments receiving seed inoculation with reference strain + *B. japonicum* and foliar spray with reference strain followed by T₇ (seed inoculation with ML₅₅ + *B. japonicum* and foliar spray with ML₅₅) both at 30 and 60 DAS. The analysis of the population dynamics of *Azotobacter* and phosphate solubilizing bacteria revealed quite interesting results. The population of all these beneficial microorganisms was found to be unaffected in presence of methylotrophs. This indicates that the methylotrophs and *rhizobia* when used in combination do not exert any inhibitory effect.

Similar results were noticed by Senthil Kumar *et al.* (2002). The *Methylobacterium* Sp. CO 47 was found compatible with all microorganisms used as bioinoculants viz., *Rhizobium*, sp. COC10, *Azospirillum lipoferum* AZ204, *Trichoderma viridae* TV6 and *Aspergillus niger*.

5.4 NUTRIENT UPTAKE (N, P AND K)

In case of nutrient uptake, inoculation treatment T₉ (reference strain + *B. japonicum*) plus foliar spray with reference strain followed by treatment receiving seed inoculation with ML₅₅ and *B. japonicum* showed higher N, P, and K uptake values when compared to control and rest of the treatments. Treatment T₇ showed maximum uptake of N, 37.0 per cent both at 30 and 60 DAS. However, the N, P uptake was lowest in treatment T₅ which is only seed inoculation with *B. japonicum*. The increase in N and K uptake due to combined inoculation of two (or) more organisms has been documented by several workers (Balamurugan and Gunasekaran, 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 inoculation with rhizobium leguminosarum by trifoli in wheat, corn, radish, mustard, rice and PPFMs in soybean respectively.

5.5 ENZYME ACTIVITIES

It is well known that enzymes play a key role in the transformation, recycling and availability of plant nutrients in soil. They are likely to be influenced by fertilizers and manures. Various enzyme activities were found to be maximum in treatment receiving both seed inoculation plus foliar spray.

Soil is a living tissue with complex biochemical reactions going on regularly. Several enzymes in soil catalyze these biochemical reactions which are responsible for nutrient cycling in soils. The enzymes that are directly concerned with carbon, nitrogen and phosphorus cycles in soils are urease, dehydrogenase and phosphatases.

In the present investigation, there was increase in dehydrogenase, urease and phosphatase activities in treatment T₉ followed by T₇ (seed inoculation with ML₅₅+*B. japonicum* and spray with ML₅₅). Both recorded maximum enzyme activities when compared with control and rest of the single dual inoculation treatments.

In the entire crop period, the enzyme activity increased initially at 30 DAS and then declined with the age of the crop. These observations are in accordance with the findings of Singaram and Kamalakumari (1995) wherein they also noticed similar trend in phosphatase activity in maize rhizosphere. The variations in the urease and phosphatase were little influenced by different inoculation treatments. More than the microbial population, the enzyme activities are regulated by the soil characters like organic carbon, pH and nutrient status (Nagaraja *et al.*, 1998).

Chendrayan *et al.* (1980) were also of the opinion that the increase in dehydrogenase activity was mainly due to the higher microbial population. The earlier studies revealed that the enzyme activities are often used as indices of microbial growth rather than the microbial number, which further may reflect the microbial respiration and the potential capacity of soil to perform biological transformations of importance to soil fertility.

6. SUMMARY AND CONCLUSIONS

An attempt was made to study the effect of combined inoculation of methylotrophs and *B. japonicum* and foliar spray of *Methylobacterium* isolates on growth, yield, nutrient uptake, enzyme activity and other beneficial organisms on soybean crop under field conditions.

The data on plant height, number of leaves, branches, nodulation, nodule dry weight, shoot and root dry weight, chlorophyll content, population of PPFM's, free living nitrogen fixers, nutrient uptake and biological transformations through enzyme activity as influenced by *Methylobacterium* and *B. japonicum* and foliar spray with respective *Methylobacterium* isolates were recorded. The results of these studies are summarized in this chapter.

All methylotrophs used in the present study along with *B. japonicum* and foliar spray with *Methylobacterium* isolates showed better performance in growth and yield attributes, nitrogen uptake and enzyme activities, which are the indicators of microbial activity in the soil. None of the isolates solubilized inorganic phosphates.

All the inoculation treatments along with *B. japonicum* and spray of *Methylobacterium* isolates enhanced better plant growth and finally contributed for better yield compared to control which was seed inoculation with *B. japonicum*.

Among the inoculation treatments, co-inoculation performed better than control, whereas, co-inoculation and foliar spray with *Methylobacterium* isolates performed better over combined inoculation with dual culture. The results of our studies indicate that the combined inoculation of methylotrophs with *B. japonicum* and foliar spray exert more favourable effect on growth and productivity of soybean than dual inoculations.

1. Treatment T₇ (seed inoculation with ML₅₅ + *B. japonicum* and FS with ML₅₅) showed 17.43 per cent increase in plant height when compared to control.
2. There was increase in nodule number both at 45 and 60 DAS in treatment receiving seed inoculation plus foliar spray compared to control which received seed inoculation only with *B. japonicum*.
3. There was increase in total dry matter of soybean plants in T₉ by 41.67 per cent when compared with control.
4. The grain yield of soybean was increased in T₉ by 38 per cent over control which received seed inoculation only with *B. japonicum* (SB₁₂₀).
5. Nutrient uptake was more in the treatment receiving both seed inoculation + foliar spray (T₉) by 37 per cent when compared with control.
6. Dehydrogenase, urease and phosphatase activities in rhizosphere soil of soybean were significantly higher in treatment T₉ at all the stages of crop growth when compared with control and all other treatments receiving seed inoculation with dual culture.

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APPENDIX I

Composition of media

Ammonium mineral salt (AMS media)

NH ₄ Cl	-	0.50 g
K ₂ HPO ₄	-	0.70 g
KH ₂ PO ₄	-	0.54 g
MgSO ₄ ·7H ₂ O	-	1.00 g
CaCl ₂ ·2H ₂ O	-	0.20 g
FeSO ₄ ·7H ₂ O	-	4.00 mg
ZnSO ₄ ·7H ₂ O	-	100 µg
MnCl ₄ ·4H ₂ O	-	30.0 µg
H ₃ BO ₄	-	300.0 µg
CaCl ₂ ·6H ₂ O	-	200.0 µg
CuCl ₂ ·2H ₂ O	-	10.0 µg
NiCl ₂ ·6H ₂ O	-	20.0 µg
Na ₂ MoO ₇	-	60.0 µg
Agar	-	18.0 g
Distilled water	-	1000 ml
pH	-	6.8
Methanol	-	0.5%

Norris N-free medium

K ₂ HPO ₄	-	1.0 g
MgSO ₄	-	0.2 g
CaCO ₃	-	1.0 g
NaCl	-	0.2 g
Glucose	-	10.0 g
Agar	-	18.0 g
Distilled water	-	1000 ml
pH	-	7.2

Beijerinckia agar medium

Glucose	-	20 g
K ₂ HPO ₄	-	0.8 g
KH ₂ PO ₄	-	0.2 g
MgSO ₄ ·7H ₂ O	-	0.5 g
FeCl ₃ ·7H ₂ O	-	0.5 g
Na ₂ MoO ₄ ·2H ₂ O	-	0.5 g
Distilled water	-	1000 ml
Agar	-	18 g
pH	-	4.5 – 5.5

Pikovskaya's medium (Pikovskaya, 1948)

Glucose	-	10.00 g
MgSO ₄ · 7H ₂ O (2.5%)	-	10.00 ml
CaCl ₂ (1%)	-	10.00 ml
Tricalcium phosphate	-	5.00 g
Distilled water	-	1000 ml
pH	-	7.0
Agar	-	18.00 g

PERFORMANCE OF METHYLOTROPHS IN SOYBEAN (*Glycine max* (L.) Merrill) UNDER FIELD CONDITIONS

MEENAKSHI B. C.

2008

Dr. V. P. SAVALGI
MAJOR ADVISOR

ABSTRACT

A field experiment was conducted during *kharif* 2007-08 at Main Agricultural Research Station, Dharwad (Karnataka) to study the effect of combined inoculation of methylo-trophs and *B. japonicum* and foliar spray of methylobacterium isolates on plant growth in soybean under field conditions.

Seed inoculation of *Methylobacterium* isolates in combination with *Bradyrhizobium japonicum* strain (SB₁₂₀) and further foliar spraying with *Methylobacterium* isolates had significant influence on different plant growth parameters.

Among the inoculation treatments, co-inoculation performed better than control, whereas, co-inoculation and foliar spray with *Methylobacterium* isolates performed better over combined inoculation with dual culture. The results of our studies indicate that the combined inoculation of methylo-trophs with *B. japonicum* and foliar spray exert more favourable effect on growth and productivity of soybean than dual inoculations. Treatment T₇ (seed inoculation with ML₅₅ + *B. japonicum* and FS with ML₅₅) showed 17.43 per cent increase in plant height when compared to control. There was increase in nodule number both at 45 and 60 DAS in treatment receiving seed inoculation plus foliar spray compared to control which received seed inoculation only with *B. japonicum*. There was increase in total dry matter of soybean plants in T₉ by 41.67 per cent when compared with control. The grain yield of soybean was increased in T₉ by 38 per cent over control which received seed inoculation with *B. japonicum* (SB₁₂₀ + reference strain) and further foliar spray with reference strain. Nutrient uptake was more in the treatment receiving both seed inoculation + foliar spray (T₉) by 37 per cent when compared with control. Dehydrogenase, urease and phosphatase activities in rhizosphere soil of soybean were significantly higher in treatment T₉ at all the stages of crop growth when compared with control and all other treatments receiving seed inoculation with dual culture.