STUDIES ON GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH SOYBEAN [*Glycine max* (L.)]

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By

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH SOYBEAN [*Glycine max* (L.)]"** *submitted by* **Mr. NAGARAJ NAIK.,** *for the degree of* **MASTER OF SCIENCE (AGRICULTURE)** *in* **AGRICULTURAL MICROBIOLOGY,** *of the University of Agricultural Sciences, Dharwad is a record of research work done by him during the period of his study in this university under my guidance and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.*

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With Soul Searching memories...

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Introduction

I. INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important legume and oil seed crop indigenous to China and introduced to India in 1950's. It occupies 71.85 million hectares of area with 154.32 million tonnes of annual world production. In India, it occupies about 6.45 million hectares of an area with a production of 6.5 million tonnes (Anon., 1999). Being well suited to black soils of transitional tract of Karnataka, it is a major crop of Belgaum, Dharwad and Bijapur districts occupying 54 thousand hectares of area with annual production of 36 thousand tonnes (Anon., 1999). 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).

Soil is a habitat for a vast, complex and interactive community of soil organisms, whose activities largely determine the chemical and physiological properties of the soil and growth of the plant. From seed germination until a plant reaches maturity, it lives in close association with soil organisms. This association is determined as rhizocoenosis. The vast majority of plant associated soil organisms inhabit the rhizosphere, defined as the zone around roots and bacterial growth is stimulated by the release of nutrients. Within the rhizosphere, there is a continuos interaction between plant roots and rhizosphere

organisms that comprise the rhizosphere. These interactions can have an important influence on plant growth. They may be viewed as neutral or harmful or beneficial.

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

The productivity of soybean is often very much influenced by effective rhizosphere microflora. The word "Rhizosphere" (Hiltner, 1904) means active part of the soil volume close to roots. In recent years there has been renewed interest in the use of rhizobacteria, which when applied to seeds, tubers or roots, are able to colonize plant roots and stimulate growth and improve crop yields. These have been termed as Plant Growth Promoting Rhizobacteria (PGPR) (Botton *et al.*, 1990) and have been shown to increase plant growth, enhance nodulation, N_2-fixation and yields. In China such rhizobacteria are known as "Yield Increasing Bacteria".

The effects of PGPR on plant growth can be mediated by the direct or indirect mechanisms. The direct effects have been most commonly attributed to the production of plant harmones such as auxins, gibberllins and cytokinins as by supplying biologically fixed nitrogen. These PGPR also affect growth of plants by indirect mechanisms such as suppression of bacterial, fungal and nematode pathogens by production of siderophores, HCN, ammonia, antibiotics, volatile metabolites etc., by induced systemic resistance and/or by competing with the pathogen for nutrients or space for colonization.

Several soil microbiologists and microbial ecologists differentiated the rhizosphere microorganisms as beneficial and harmful according to their functions. Beneficial microorganisms are those that can promote plant growth by fixing atmospheric nitrogen, decomposing organic wastes and residues and enhance nutrient cycling, detoxifying pesticides, suppressing plant diseases and soil borne pathogens and by producing plant growth promoting substances such as IAA, GA, vitamins, harmones, enzymes etc. Using some of these beneficial microorganisms, various microbial inoculants have been prepared for use in crop production to reduce the cost of chemical fertilizers and to minimize environmental pollution. Since, microorganisms are useful in eliminating the problems associated with the use of chemical fertilizers and pesticides,

they are now widely applied in sustainable nature farming and organic agriculture (Higa, 1991 and Parr *et al.*, 1994).

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 inoculants have been found to perform better than single inoculations (Alagawadi and Gaur, 1988; Patil *et al.*, 1992; Jisha and Alagawadi, 1996; Prathibha *et al.*, 1994) and also reported that, they exert a moiré 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 inhabitants 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 *Rhizobium, Azospirillum,* phosphate solubilizers and bacteria isolated from rhizosphere of soybean grown in different locations of Karnataka, over single inoculation on the soybean crop with the following objectives.

1. Isolation of PGPR strains from soybean, grown in different regions of Karnataka.

2. Screening the isolates for beneficial traits like production of plant growth promoting substances and bio-control potential etc.

3. Selection of efficient isolates based on their beneficial traits.

4. Study of combined inoculation of PGPR with Rhizobial inoculants on growth and productivity of soybean.

Review of Literature

II. REVIEW OF LITERATURE

There are several beneficial rhizobacteria in the rhizosphere, which can improve soil quality, enhance crop production and protection, conserve natural resources and ultimately create more sustainable agricultural production and safe environment. Effective techniques have been developed to isolate and enumerate these organisms from the rhizosphere of crop plants and test their efficiency for beneficial effects on soil and plant as well. Literature pertaining to utilizing of these beneficial organisms as bioinoculants in agricultural crop production and their effects either as single or mixed inoculants on crop growth, yield and nutrient uptake have been reviewed in this chapter.

2.1 Isolation of PGPRs and Inoculation response of PGPRs

2.1.1 Nitrogen fixers as PGPR

2.1.1.1 *Rhizobium*

Vincent (1970) and Roughly (1970) isolated pure cultures of *Rhizobium* by washing and surface sterilizing the nodules and then plating the milky fluid from a crushed nodule on a suitable agar medium.

Dhilllon *et al.* (1974) isolated ten strains of *Rhizobium* from the nodules of bengalgram and soybean and identified

them to be *R. phaseoli* and *R. japonium*, respectively after carrying out the various biochemical tests. Patil *et al.* (1974) isolated 14 strains of *Rhizobium* from soybean grown in different parts of Karnataka and tested for nodulation in soybean through these plant passages.

Fuhrmann (1990) collected 360 isolates of *B. japonicum* from soybean nodules from 18 locations and characterized serologically with an ELISA, morphologically by colony type on yeast extract mannitol agar medium. Thompson *et al.* (1991) isolated over 1500 root nodule bacteria from traditional soybean growing areas in Thailand and reported that most isolates were slow growers. Bromfield and Jones (1980) reported that many fast growing rhizobia produce acids from sugars when grown in culture medium while slow growers produce alkaline metabolites.

A significant increase in seed yield of two pigeonpea cultivars by seed inoculation with *Rhizobium* strain KA-1 and the inoculation efficiency increased with applied P reported by Gupta and Bajpai (1981). In a nodulation study of 12 greengram genotypes including Pusabaisaki as one of the genotypes on inoculation with *Rhizobium* at different growth stages reveal that number of nodules in cultivars Pusabaisaki go on increasing upto 50 DAS and there after decreased to 19 per cent at harvest. There was a similar trend with nodulation

and nodule dry weight in other cultivars reported by Dhandopani and Sakharam Rao (1988). Highest nodulation and highest yield in soybean was recorded when a treatment including *Rhizobium* in combination with a nitrogen fertilizer was applied.

Parashar *et al.* (1999) conducted a field trial to know the effect of *Rhizobium* inoculation and phosphorus application on quality of broadbean (*Vicia faba* L.) and opined that *Rhizobium* inoculation with application of 60 kg P_2O_5 ha^{-1} caused marked increase in seed yield and quality parameters of broad bean seeds such as protein, methionine and tryptophan.

Sharma *et al.* (1999) studied the response of 11 strains of *Rhizobium* and nitrogen on symbiotic, biochemical and physiological parameters and yield of black gram (*Vigna mango* (L.) Hepper) and noticed the highest yield of black gram in local strain × N 20 kg ha^{-1} treatment combination.

2.1.1.2 *Azospirillum*

Agarwal and Tilak (1988) studied the establishment of *Azospirillum brasilense* in the roots of *Eleusine coracana* and reported the establishment of bacteria endobiotically in roots, mainly in the cortical intracellular space and vascular tissue. Al-Maadhidi (1989) isolated *Azospirillum* from the roots of wheat cultivars and identified them as *Azospirillum brasilense*

and *A. lipoferum* and noted *A. brasilense* as the most potent nitrogen fixing species. Jaskowska (1995) investigated the occurrence of bacteria in the rhizosphere of rye, wheat, barley and corn and found *Azospirillum* in 51 per cent of the samples. Maheshkumar (1997) isolated *Azospirillum* from the roots of *Bombusa vulgaris* and *Dindrocalamus strictus* grown on different soils.

Rao *et al.* (1985) carried out field experiment at various location in India on response of *Azospirillum brasilense* and demonstrated that grain yield of sorghum (*sorghum bicolor*) pearlmillet (*Pennisetum americanum*), fingermillet (*Eleusine coracana*) and barley (*Hardeum vulgare*) increased, either with or without urea and *Azospirillum* seed inoculation. In a pot culture experiment Gallo *et al.* (1990) found that chickpea plants inoculated with *Azospirillum brasilense* produced greater root and shoot dry weight and root length than those inoculated with *Rhizobium leguminosarum.*

Maheshkumar *et al.* (1997) reported improved seedling growth of bamboo due to inoculation of *Azospirillum* sp. Increased plant weight, grain and fodder yield and savings of 25 per cent N fertilizer in *rabi* sorghum due to inoculation of *Azospirillum* sp., was observed by Tippannavar and Alagawadi (1998). Alagawadi and Krishnaraj (1998) reported increased

grain yield in sorghum due to inoculation of *Azospirillum* strains ACD-15 and ACD-20.

2.1.2 Phosphate solubilizers

As early as 1948, Pikovskaya obtained from the soil and phosphorus bearing rocks, an organism which was termed as "Bacterium P" capable of forming water soluble phosphate from tricalcium phosphates.

Phosphate solubilizing microorganisms belonging to the genera *Bacillus*, *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Rohnella* and *Serratia* have been isolated in the recent years from the soils and rhizosphere of crop plants by various workers (Halder *et al.*, 1990; Krishnaraj and Gowda, 1990; Illmer and Schinner, 1992; Leinhos and Vacek, 1993; Kim *et al.*, 1998 and Maheshkumar, 1997).

Bopaiah (1985) isolated phosphate solubilizing bacteria of the genera *Bacillus* and *Pseudomonas* from the rhizosphere of arecanut palm. Molla *et al.* (1984) reported active phosphate solubilizing microbial population in the rhizosphere of rye grass and wheat. Yahya and Al-Azawi (1989) enumerated phosphate solubilizing bacteria in 52 soil samples of Bhagdad where the phosphate solubilizing bacteria varied from 0.012 to 28.4 $\times$ 10^4 cells g^{-1} of soil.

Rokade and Patil (1993) conducted seed as well as soil inoculation with phosphate dissolving microorganisms (PDMOs) and found them to be effective in increasing yield and dry matter content of different crops. Chanway (1995) studied the differential response of western hemlock (*Tsuga heterophylla* (Raf.) sarg) with plant growth promoting *Bacillus polymyxa* strain L6-16R and obtained significant increase in seeding emergence height and biomas accumulation.

Defreitas *et al.* (1997) inoculated phosphate solubilizing rhizobacterium to Canolo (*Brassica napus* L.) and obtained significant increase in plant height and pod yield of canola, but P-uptake was not increased.

Highest number of double seeded and filled pods and increased pod weight, 100 kernel weight, shelling percentage, pod, kernel, oil and protein yield were obtained in treatment which received MRP (75%) + SSP (25%) along with nine tonnes of FYM ha^{-1} and *Aspergillus awamori*. The pod yield in this treatment was 40 per cent higher than the one with only MRP (75%) + SSP (25%) alone (Ramesh *et al.*, 1998).

2.2 Screening of PGPRs

2.2.1 *Rhizobium*

Erdman and Means (1952) studied the several characteristics used for measuring nitrogen fixation of

atmospheric nitrogen by inoculated Rhizobia and recorded a positive correlation between total yields, dry weight of the plants and nitrogen content of the plants.

Certain desirable characters for use and selection of efficient *Rhizobium* strain were suggested by Bergerson *et al.* (1971). Lahiri (1979) suggested that the differential competition between the strains of *Rhizobium* in nodulating soybean is the resultant expression of two interaction forces operating simultaneously, like preferentially may be exploited by the particular bacterial strain. The magnitude of second compound depends more on the nature of strains and could be manipulated to some extent artificially.

Lopes and Giardini (1974) reported that the nodulation and the total plant dry weight was not as a good criterion as the total nitrogen for strain differentiation although total nitrogen and dry weight are correlated.

Twenty isolates of horsegram were screened for their symbiotic response in pots UASB-87 strain for horsegram was developed which was efficient and effective in various parts of Karnataka (Siddaramaiah, 1977).

Thomas and Shantaram (1987) screened *Rhizobium* isolates of eight different forage legumes based on nodulation

and nitrogen fixing efficiency and compared with NiftAL composite cultivars.

2.2.2 *Azospirillum*

The nitrogen fixing efficiency of *Azospirillum* species has been examined by several workers.

Dobreiner and Day (1976) reported *Azospirillum* to fix as much as 150 mg of N/g of lactate utilized. However, nitrogen fixation values equivalents to this have not been observed by other workers.

Okon *et al.* (1977) reported 20-24 mg of N fixed per gram of carbon source utilized whereas Nelson and Knowels (1978) observed 4.7 to 28.0 mg N/g of carbon source. Dobereiner and Boddey (1980) reported that the efficiency of nitrogen fixation increased with increasing age of culture reaching values of 98 mg/g of lactate and 49 mg/g of glucose for *A. brasilense* and *A. lipoferum* respectively in the early stationary phase.

Purashothaman *et al.* (1988) reported that nitrogen fixing potential of *Azospirillum* sp., varies between 1.6 and 23.96 mg/g of carbon source. Prathibha (1993) observed 2.40 to 18.28 mg of N/g of carbon source utilized by 25 *Azospirillum* strains isolated from 14 genotypes of cotton. Maheshkumar (1997) isolated six *Azospirillum* strains from the roots of

bamboo plants and found them to fix 15.68 to 22.4 mg of N/g of carbon source utilized.

2.2.3 Phosphate solubilizers

Oswal and Bhide (1972) reported that *Pseudomonas* sp., isolated from Maharashtra soils could solubilize 13-58 per cent of tricalcium phosphate added to liquid medium. Sardina *et al.* (1986) reported a 50 per cent solubilization of phosphorus from low grade mineral residues by *Pseudomonas* sp., when they were added at the rate of 1 g/l of medium. Gaur (1990) has reported a range of 24 to 58.4 per cent solubilization of phosphorus from tricalcium phosphate.

Singh and Kapoor (1994) observed increased in solubilization zone of tricalcium phosphate and mineral rock phosphate on inoculation with phosphate solubilizing bacteria upto 15 days in plates due to increase in the level of glucose 1 to 2 per cent. Nahas (1996) studied 31 bacteria isolated from soil for their ability to solubilize rockphosphate and calcium phosphate in culture medium and found eight bacteria to possess solubilizing ability. Among the isolates, *Pseudomonas cepacia* showed highest solubilizing activity. Maheshkumar (1997) recorded maximum solubilization of tricalcium phosphate by *Serratia* sp., on 15th day of incubation.

Venkateshwaralu *et al.* (1984) and Gaur (1990) showed that production of organic acids by phosphate solubilizing is one of the mechanisms to solubilizing insoluble mineral phosphate. Solubilization of tricalcium phosphate is also reported to occur even in the absence of release of organic acids (Illmer and Schinner, 1992). Goldstein *et al.* (1993) showed that solubilization of mineral phosphates by bacteria is the result of acidification of the periplasmic space by the direct oxidation of glucose or other aldose sugars.

Organic acid production may play an important role in hydroxy apatite solubilization but is not the sole reason for increased phosphorus concentration in the culture medium (Kim *et al.*, 1998).

2.3 Production of growth promoting substances by PGPRs

2.3.1 *Rhizobium*

Production of indole acetic acid and other indole compounds by *Rhizobium* sp., were reported from root nodules of leguminous tree *Pongamia pinnata* (L.) Pierre. The *Rhizobium* sp., isolated was able to produce IAA in culture when supplemented with L-tryptophan @ 110 µg/ml (Sinha and Basu, 1981).

Rhizobium sp., form the root nodules of a leguminous medicinal herb (*Tephrosea purpurea* Pers.) were isolated by De

and Basu (1996) that produced high amounts of indole acetic acid (126 µg/ml) from L- tryptophan supplemented basal medium and reported maximum IAA production in the stationary phase of growth of the bacteria.

Chhaya Datta and Basu (1997) isolated a *Rhizobium* sp., from the root nodules of a leguminous medicinal herb (*Dolichos biflorus* L.) and found to produce large amounts of indole acetic acid (139.1 µg/ml) from L-tryptophan and also discussed the possible role of the rhizobial production of IAA on the rhizobia legume symbiosis.

2.3.2 *Azospirillum*

Plant growth promoting substances released by *Azospirillum* isolated from rhizosphere of crop plants was reported by Harari *et al.* (1983).

Fallik and Okon (1984) identified the growth promoting substances produced by *A. brasilense* as indole acetic acid. Bar and Okon (1993) reported that the phyto hormone IAA was produced by *Azospirillum* and the organism promoted the plant growth.

Omay *et al.* (1992) also observed the production of IAA by *Azospirillum* sp., under different growth conditions. Hernandez *et al.* (1996) observed *Azospirillum* isolates to produce IAA

within the broth and suggested that the organism increased biomass accumulation of *Panicum maximum*.

2.3.3 Phosphate solubilizers

Phosphate solibilizing microorganisms are also known to produce plant growth promoting substances.

Barea *et al* (1976) showed the production of plant growth regulators by rhizosphere phosphate solubilizing bacteria. They isolated about 50 phosphate dissolving bacteria from rhizosphere of crop plants and examined for IAA, gibberellins and cytokinin production, 43 of these isolates produced IAA, 29 formed gibberllins and 45 produced cytokinin like substances.

Baya *et al.* (1981) reported the production of vitamin by rhizosphere isolates and found a direct correlation with solubilization of different phosphates. Sattar and Gaur (1987) tested eight phosphate solubilzing bacteria isolates and found them to synthesize auxins and gibberlins. Phosphate solubilizing microorganisms are also reported to produce siderophores and auxins (Chabot *et al.*, 1993; Krishnaraj, 1996). De freitas *et al.* (1997) recorded significant increase in plant height and pod yield of canola (*Brassica napus* L.) due to inoculation with phosphate solubilizing rhizobectrium but P uptake was not increased by the organism.

2.4 Plant growth promoting rhizobacteria

The free living soil bacteria that provide some kind of benefit to plants are usually referred to as plant growth promoting rhizobacteria are PGPR (Kloepper *et al.*, 1989) or Yield Increasing Bacteria YIB (Piao *et al.*, 1992; Tang, 1994). A number of bacteria including *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia* and *Bacillus* can be considered as plant growth promoting rhizobacteria (Brown, 1974; Kloepper *et al.*, 1988; Bashan and Levanony, 1990; Tang, 1994; Okon and Labandera-Gonzalez, 1994). Plant growth promoting rhizobacteria can effect plant growth either directly or indirectly. There are several ways in which different plant growth promoting rhizobacteria have been reported to directly facilitate the proliferation of their plant hosts. They can fix atmospheric nitrogen and supply it to plants, synthesise siderophores that sequester iron from the soil and provide it to plants, synthesise several different phytoharmones that can act to enhance various stages of plant growth, they have mechanisms for the solubilzation of minerals such as P and make it readily available for plant uptake and they may synthesize some low molecular weight compounds or enzymes that can modulate plant growth and development (Brown, 1974; Kloepper *et al.*, 1986 and 1989; Davison, 1988; Lambert and Joos, 1989; Glick *et al.*, 1994a and 1994b).

Yoshikawa *et al.* (1993) reported that secretion of succinic and lactic acids per se by a plant growth promoting rhizobacterial strain *Pseudomonas putida* stimulated root growth in *Asparagus* seedlings.

Chanway (1995) reported that seed inoculation of western hemlock (*Tsuga hecterophylla*) with plant growth promoting *B. polymyxa* strain L6-16R can result in significant increase in the seedling emergence, height and biomass accumulation.

2.5 Dual inoculation effect on crop plants

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

Combined inoculation of *Rhizobium* and *A. chroococcum* or *Beijerinckia indica* results in improved nodulation and increase in yield in soybean (Apte and Iswaran, 1975; Kumar Rao and Patil, 1976; Rawat and Sanoria, 1976; Sharma and Rao, 1978), in chickpea (Tosh and Sanoria, 1978), in mungbean, soybean and pea (Jauhri *et al.*, 1979) and in soybean, *Vigna* and *trifolium* (Burns *et al.*, 1981) have been observed.

Inoculation of *Azospirillum* is also reported to show the improved nodulation and yield of soybean in unsterile soil (Sing and Subba Rao, 1979). Similar results have been recorded by

Tilak *et al.* (1981) in clover, Lucerne and chickpea inoculated with both *Azospirillum* and *Rhizobium* simultaneously.

Inoculation of chickpea with *A. brasilense* together with *Rhizobium* is known to increase the grain yield, nodule dry weight, nitrogen activity and active iron content of nodules (Rai, 1983).

Plazinski and Rolfe (1985) observed inhibition of nodulation in clover plants due to combined inoculation of *Rhizobium trifolii* and *Azospirillum* on addition of auxins to the plant growth medium, but an Hac strain of *Rhizobium trifolii* was still able to form nodules in the presence of *Azospirillum*.

Galal (1997) reported that dual inoculation with *Bradyrhizobium japonium* and *Azospirillum brasilense* improved the crop growth and biological nitrogen fixation of soybean over single inoculation.

Seed inoculation with *Azospirillum brasilense* and *Klebsiella pneumoniae* in combination with application of 60 kg N/feddan gave slightly higher dry matter and protein yields in pearlmillet than application of 90 kg N/feddan alone or single inoculation of each organism (Mahmoud *et al.*, 1994).

A significant increase in root nitrogen activity, dry matter and seed yields of rice and sorghum due to combined inoculation of *A. brasilense* and phosphate solubilizing *P.*

striata or *Bacillus polymyxa* as compared to single inoculation of either organism or control (Gaur and Alagawadi, 1987).

Umamaheswar Rao and Rao (1993) reported associative effect of *Rhizobium* with *Glomus mosseae* or *G. epigaeum* on blackgram and greengram and showed significant increase in growth, N and P uptake, nodulation and biochemical constituents like leaf chlorophyll, total soluble sugars, total phenols and free amino acids when compared to inoculation of *Rhizobium* alone.

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 and among *Azospirillum* isolates ACR-8 (local isolate) showed better performance than SP-7.

Inoculation of selected plant growth promoting rhizobacteria *Pseudomonas* and *Rhizobium* colonized the rhizosphere of wheat rape, pea, alfalfa and sugarbeat in a pot culture experiment, but no clear growth stimulation effect was observed (Hoflich *et al.*, 1995).

Thakur and Panwar (1997) in a field experiment studied the response of PS 16 and PUSA 105 greengram varieties to *Rhizobium* and VAM fungi inoculation. Nodulation, nitrate

reductase and nitrogenase activity was higher in dual inoculation plants than single inoculated PUSA 105 variety showed higher activity than PS 16.

Alagawadi and Gaur (1992) also revealed a significant increase in yield and nutrient uptake of sorghum due to combined inoculation of *A. brasilense* and *P. striata* or *B. polymyxa* over inoculation of individual organisms.

A significant increase in the yield of red cotton was recorded by Prathibha *et al.* (1994) due to combined inoculation of *Azospirillum* and *P. striata* as compared to single inoculation treatment.

Dual inoculation of *Rhizobium* and phosphate solubilizing bacteria have been shown to increase the nodulation nitrogen fixation, nutrient uptake as well as yields of mungbean, soybean, clover, commonbean and other crops (Bhatnagar *et al.*, 1973; Delorenzini *et al.*, 1979; Grimes and Mount, 1984).

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. polymyxa* as compared to single inoculation treatments.

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 gardenpea compared to recommended levels of nutrients applied through chemical fertilizers only.

Prabhakaran *et al.* (1999) showed the dual inoculation of *Rhizobium* and phosphate with 50 per cent of P_2O_5 per hectare recorded a comparable yield with 100 per cent P_2O_5 per hectare alone application in horsegram which indicated saving of 50 per cent of chemical fertilizers of phosphorus due to inoculation.

Dual inoculation with *Glomus mosseae* and *Rhizobium* with 50 per cent of recommended N and P fertilization recorded the maximum plant height, root length, plant biomass, number of nodules per plant, nodule weight and grain yield of greengram variety Vamban compared to single inoculation or 100 per cent recommended dose of fertilizer application. Mycorrhization positively influenced *Rhizobium* for better nodulation and N_2 fixation (Balachander and Nagarjan, 1999).

2.6 Effect of multiple inoculation on crop plant

El-Mukkadam *et al.* (1989) observed improved nodule branching due to combined inoculation of *Azospirillum*

lipoferum, Azotobacter chroococcum and *Rhizobium* spp., as compared to dual inoculation treatment.

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

El-Demerdash *et al.* (1992) observed highest nitrogenase activity in rhizosphere and grain yield in wheat plants inoculated with mixed cultures of cowpea rhizobia, *Rhizobium leguminosarum, Azotobacter chroococcum* and *Azospirillum lipoferum* as compared to control and other inoculation treatments.

Belimov *et al.* (1995) found that the mixed cultures of *Azospirillum lipoferum* 137, *Arthrobacter mysorens* 7 and phosphate solubilizing *Agrobacterium radiobacter* 10 produced a significant positive effect on the grain yield of barley. They suggested that the combined inoculation of all the three organisms provided a more balanced nutrition for plants and the improvement in root uptake of N and P was a major mechanism of interaction between plants and bacteria.

In a pot culture trial conducted by Veena (1999) reported maximum plant growth, biomass yield and nutrient uptake in sorghum in the treatment receiving consortia of eight component organisms comparing bacteria, fungi, actinomycete

which was almost equivalent to application of 75 to 100 per cent recommended dose of chemical fertilizers. Combined inoculation of four beneficial organisms was also found superior over single, dual or triple inoculation of beneficial organisms and also a field experiment was conducted by Devananda (2000) reported maximum plant growth, yield and nutrient uptake in pigeonpea in the combined inoculation treatment comprising of *Rhizobium*, *Azospirillum* and *P. striata* (PGPRs).

Materials & Methods

III. MATERIAL AND METHODS

The present study was carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, during the years 1999-2000 and 2000-01. The material used and the methods followed in carrying out the experiments are presented here.

3.1 Collection of soybean plants and rhizosphere soils

A survey for the soybean root nodulation was undertaken in the two Agro-climatic regions *viz.*, II and IV, which include Northern dry zone No. 3 and transition zone No. 8. The soybean plants approximately 40-50 days old were collected from 30 locations where soybean is predominantly grown.

The healthy soybean plants were randomly selected for the study from each field in each location as mentioned in Table 1. The plants were uprooted carefully without disturbing the root system. The extent of nodulation as number of nodules and nodule weight were observed. The plants were dried in hot air oven for dry weight.

Collection of soil samples

The rhizosphere soils that adhere to the soybean plants were collected and were brought in polythene bags and stored in a cool spot and analysed immediately.

3.1.1 Soil chemical characters and nodulation status

Detailed chemical analysis of 30 soil samples were carried out as explained below (Table 1).

3.1.1.1 Soil pH

The pH of the soil water suspension (1:2.5) was determined using a digital pH meter with combined glass electrode (Jackson, 1967).

3.1.1.2 Electrical conductivity

The EC was determined in 1:2.5 soil water extract using electrical conductivity bridge (Jackson, 1967).

3.1.1.3 Cation exchange capacity

The cation exchange capacity (CEC) of the soil was determined by leaching the soil with neutral normal sodium acetate solution and the excess salts were removed by 60 per cent ethanol solution. The adsorbed sodium was replaced by neutral normal NH_4OHC and the concentration of sodium in the leachate was measured by flame photometer and the cation exchange capacity was calculated (Jackson, 1967).

3.1.1.4 Available nitrogen

Available nitrogen was estimated by micro-kjeldhal method (Black, 1965). Soil sample was digested in H_2SO_4

containing salicilic acid, K_2SO_4, $CuSO_4$ and Na_2SO_3. This was distilled with excess of 40 per cent NaOH. The ammonia released was absorbed in 4 per cent boric acid. Nitrogen was determined by back titration against H_2SO_4 using mixed indicator.

3.1.1.5 Available phosphorus

Available phosphorus was estimated by Olsen's method (Muhr *et al.*, 1965). Soil sample was shaken with the extractant for few minutes, filtered and phosphorus in an aliquat of the extract was determined by chlorostanous reduced molybdophospheric blue colour method in HCl system. The intensity of the blue colour was read in a spectophotometer at 660 nm wave length.

3.2 Isolation of rhizobacteria

3.2.1 Isolation of *Rhizobium* strains from soybean root nodule

Soybean plants collected along with root system in polythene bags were packed properly to retain the nodules fresh. The nodules washed in tap water were carefully detached from the root system and surface sterilized in 70 per cent alcohol after dipping in 0.1 per cent mercuric chloride solution for three minutes. These nodules were thoroughly washed in 6-8 changes of sterile distilled water. Big, healthy pink nodules

Table 1. Chemical characteristics of the soil.

Sl. No.	Place	Soil type	PH	EC (dSm^{-1})	CEC (C mol kg^{-1})	Avail N (kg ha^{-1})	Avail P (kg ha^{-1})
1	Agadi	Black	6.8	0.20	44	131.00	23.57
2	Algur	Red	7.9	0.50	36	178.52	22.24
3	Achatageri	Red	6.2	0.90	30	195.57	21.06
4	Ainapur	Red	6.3	0.35	49	201.00	17.21
5	Athani	Black	7.4	1.50	41	251.52	17.04
6	Bijapur	Black	8.2	0.70	49	145.00	28.80
7	Chikkodi	Black	8.1	0.34	59	274.54	23.85
8	Dyamapur	Red	6.3	0.20	21	173.00	12.11
9	GTC DWD	Red	6.8	0.52	33	170.00	15.06
10	Hanumanamatti	Red	6.9	0.31	28	191.02	18.06
11	Haroogeri	Black	7.5	0.44	55	206.58	26.08
12	Hirehonnalli	Black	7.2	3.20	49	224.64	24.80
13	Honnawad	Black	7.6	0.88	45	183.00	14.60
14	Hooli	Yellow	5.2	0.75	22	112.00	14.00
15	Hubli	Black	7.2	0.85	55	163.55	25.28
16	Koulgudda	Black	7.6	2.55	54	224.00	19.81
17	Misrikoti	Black	7.3	0.10	54	216.32	22.00
18	Mole	Black	7.5	1.25	45	251.32	17.90
19	Mudhol	Black	6.8	0.19	48	275.00	26.80
20	Nippani	Black	7.8	0.50	49	181.92	9.80
21	Padarangh	Black	7.4	0.20	41	210.82	19.62
22	Pale	Red	6.8	0.50	35	196.82	19.72
23	Polekoppa	Red	6.9	0.90	20	163.41	9.80
24	Ramdurga	Black	7.1	1.50	53	248.44	22.32
25	Sankeshwar	Black	7.9	0.70	63	198.16	23.86
26	Saundatti	Black	7.5	0.34	44	154.61	14.80
27	Seed farm Athani	Red	6.5	0.20	32	218.32	17.84
28	Telsang	Black	8.2	0.52	42	216.15	15.50
29	UAS, DWD	Black	8.1	0.31	48	174.80	31.85
30	Ugar	Black	7.9	0.44	55	272.40	22.20

Table 2. Characters of plant collected from different locations.

Sl. No.	Place	Nodule number plant^{-1}	Nodule dry weight (mg plant^{-1})	Plant dry weight plant^{-1} (g)
1	Agadi	27.66	55	16.4
2	Algur	26.50	106	17.3
3	Achatageri	27.67	53	16.2
4	Ainapur	61.33	234	19.2
5	Athani	65.00	260	18.4
6	Bijapur	48.95	213	17.2
7	Chikkodi	52.95	217	19.8
8	Dyamapur	13.75	31	14.9
9	G.T.C. DWD	25.99	75	16.5
10	Hanumanamatti	69.50	278	18.8
11	Haroogeri	25.99	86	19.2
12	Hirechonalli	20.00	41	11.6
13	Honnawad	58.67	234	19.2
14	Hooli	10.99	29	15.8
15	Hubli	68.33	136	14.3
16	Koulgudda	32.66	110	16.2
17	Misrikoti	84.47	165	20.2
18	Mole	39.00	129	16.8
19	Mudhol	25.93	79	17.3
20	Nippani	26.50	73	16.3
21	Radarsang	37.62	147	13.5
22	Pale	16.33	33	14.8
23	Polekoppa	24.50	87	19.2
24	Ramdurga	66.00	128	17.5
25	Sankeshwar	58.67	191	19.8
26	Saundatti	39.00	111	16.2
27	Seed form Athani	16.33	65	15.2
28	Telsang	52.95	216	20.2
29	UAS, DWD	70.50	272	20.6
30	Ugar	41.99	126	17.5

were crushed with sterile glass rod to get the nodule suspension. A loopful of white milky nodule suspension was streaked on plates of yeast extract mannitol agar containing congored (2.5 ml/l of 1%) as per the method described by Hahn (1966). These plates were incubated for four to seven days at 26-28°C in a incubator. The colonies showing little or no congored absorption were further purified by the streak plate method. Well isolated single colonies on the plates were isolated and preserved in YEMA slants.

3.2.2 Isolation of *Azospirillum* from soybean roots

Root samples of soybean plants collected from different locations were washed thoroughly in running tap water. The roots were then cut into bits of 1-2 cms length. These root bits were then surface sterilized by dipping in 0.1 per cent $HgCl_2$ solution for three minute and then in 70 per cent ethyl alcohol for one minute followed by washing in 6-8 changes of sterile water. Then root bits were inserted into the test tubes containing sterilized semisolid N-free malate medium (Okon *et al.*, 1977). The tubes were incubated for one week and observed for growth of *Azospirillum* as sub surface white pellicle. The isolates were purified by repeated sub-culturing in fresh tubes followed by streaking on potato infusion agar (Baldani and Dobereiner, 1980) till distinct pure colonies were obtained.

3.2.3 Isolation of phosphate solubilizing bacteria and general rhizobacteria from rhizosphere soil

The roots of soybean plants were gently tapped to remove excess soil and soil adhered to the root was collected and used for isolation of microflora by serial dilution plate count method. For phosphate solubilizing bacteria, Pikovskay's agar medium (Pikovskaya, 1948) and for General rhizobacteria, soil extract agar (Bunt and Rovira, 1955) were used. The plates were incubated at 28±2°C for four to seven days and colony counts recorded. Predominant colonies of each group of organism were purified and subcultured on the slants of respective media for further use.

3.3 Identification of the isolates

The rhizobacteria isolated from soybean were identified upto generic level based on morphological and biochemical tests as detailed below.

3.3.1 Identification of *Rhizobium* strains

The pure cultures of nodule isolates were subjected to the following determinative tests.

3.3.1.1 Growth on YEMA + congored medium

The procedure as described by Vincent (1970) was followed. YEMA medium with congored (2.5 ml of 1% solution) was prepared and 15 to 20 ml of this medium was dispensed in petriplate. Young cultures of collected purecultures were streaked on these plates and incubated for 7 days at 26-28°C. Little or no absorption of congored indicates the presence of *Rhizobium*. The number of days for the colonies to appear and the nature of the colonies formed were also recorded. The production of polysaccharide was also observed.

3.3.1.2 Gram reaction

Gram staining for the isolates was conducted following the procedure described by Anonymous (1957). Typical gram negative short rods were inferred to be rhizobia.

3.3.1.3 Acid or alkali production

Freshly prepared YEMA plates containing Bromo Thymol Blue (BTB) having a pH of 7.0 were streaked with bacterial isolates as per the method described by Vincent (1970). Slow growing rhizobia produce alkalic metabolites in this medium which is indicated by change in the colour of BTB dye, from green to blue while fast growers were identified by the production of acidic metabolites changing colour of dye from green to yellow.

3.3.1.4 Growth on glucose peptone agar medium

The method described by Vincent (1970) was followed. Young cultures were streaked on to freshly prepared glucose peptone agar plates and incubated at 26-28°C for 7 days. Poor growth in the medium was confirmatory test for *Rhizobium*.

3.3.1.5 Growth on Hofer's alakali broth

The above test was conducted as a confirmatory test for *Rhizobium* as per the procedure described by Hofer (1935). The pH of YEMA broth was raised to 11.0 by adding sodium hydroxide. The broth was then dispensed into test tubes at the rate of 5 ml per tube and autoclaved at 121°C and 1.06 kg pressure for 15 minutes. The isolates were inoculated into these tubes and incubated at 28°C for 7 days. Inability of these isolates to grow on YEM broth at pH 11 was confirmatory for *Rhizobium*.

3.3.1.6 Ketolactose test

Retest isolates were streaked on petriplates containing ketolactose medium and the plates were incubated at 26-28°C for 7 to 8 days in a incubator. After the incubation period, the plates were flooded with Benedict's solution. The change in colour from blue to yellow around the colonies indicates

conversion of lactose and tests were considered positive for *Rhizobium* (Bernaerz and Deley, 1963).

3.3.2 Efficiency test of isolates

3.3.2.1 Nodulation test

To study the efficiency of different isolates the modified experiment of N free sterile sand media in plastic cups was followed as described by Nambiar and Dart (1980).

3.3.2.2 Preparation of assembly

Plastic cups with proper drainage were used for the test. The plastic cups were washed with two per cent formaldehyde and filled with 600 grams of acid washed sterilized, dried river bed sand aseptically.

3.3.2.3 Plant nutrient solution

The details of plant nutrient solution and composition is given in Appendix-I. The N-free nutrient solution applied alternate day to the cups.

3.3.2.4 Sowing of seeds

Healthy, clean uniform sized seeds of soybean (JS-335) were surface sterilized with 70 per cent alcohol followed by 0.1 per cent mercuric chloride for three minutes, than followed by washing in several changes in sterile distilled water. The

surface sterilized seeds were then germinated in petriplates containing moist filter paper. The seeds were allowed to sprout. Two sprouted seeds were sown in plastic cups containing sand and watered with sterile distilled water and plant nutrient solution alternatively. After some days only one seedling per cup was retained.

3.3.2.5 Inoculation

All the 24 isolates grown on YEMA slopes were inoculated into sterile YEM broth and incubated for 7 days. One ml of the broth culture was poured around the seedling soon after germination of the seed with the help of sterile pipette. Three replications were maintained for each treatment. The control plants were not inoculated.

3.3.3 Identification of *Azospirillum* isolates

Azospirillum isolates were streaked on N-free malate medium plates (Kreig, 1976) containing 50 mg yeast extract per litre. They were then streaked on potato infusion agar plate (Baldani and Dobereiner, 1980) and simultaneously tested for colony morphology on nutrient agar medium (Anon., 1957). The microscopic observations were made for cell shape gram reaction and motility.

3.3.4 Identification of phosphate solubilizing bacteria

The isolates of phosphate solubilizing bacteria were identified upto generic level based on morphology and biochemical testes as given below.

3.3.4.1 Morphological characters

P-solubilizing bacterial cultures isolated were tested for colony morphology, gram reaction, cell shape and ability to form spores as per the standard procedures given by Anon. (1957) and Barthalomew and Mittewer (1950).

3.3.4.2 Biochemical tests

3.3.4.2.1 Hydrolysis of starch

The capacity of the isolates to hydrolyse starch was tested by the procedure given by Eckford (1927). Triplicate plates of starch agar were inoculated with test cultures and incubated at 30°C for 3 days. After incubation the plates were flooded with Lugol's iodine solution, allowed to stand for 15-30 minutes and observed for formation of clear zone around the colony to indicate hydrolysis of starch. (Starch agar was prepared by suspending one gram of starch powder in 10 ml of cold distilled water, mixed with 90 ml of nutrient agar and autoclaved at 121°C for 20 minutes).

3.3.4.2.2 Gelatin liquefaction

The gelatin liquefaction ability of the P-solubilizing bacterial isolates was examined by the procedure of Blazevic and Ederer (1975). Triplicate plate of gelatin agar inoculated with cultures in one pot were incubated at 30°C for three days. After incubation the plates were flooded with 12 per cent $HgCl_2$ solution and allowed to stand for 20 minutes and observed for clear zone around the growth of organism to indicate gelatin liquefaction.

3.3.4.2.3 Catalase test

Nutrient slants were inoculated with test cultures and were incubated at 30°C for 24 hours. After incubation, the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles indicates positive for catalase (Blazevic and Ederer, 1975).

3.3.4.2.4 Acid and gas production

Test cultures were examined for acid and gas production by following the procedures given by Seeley and Vandemork (1970). Test cultures were inoculated to five ml of pre-sterilized glucose broth medium in test tubes containing Durham's tube and bromocresol purple (15 ml L^{-1} of 0.04% solution) as pH indicator. The tubes were incubated for seven days at 30°C. The

accumulation of gas in the Durham's tube was taken as positive for gas production and change in colour of medium to yellow was taken as positive for acid production.

3.3.4.2.5 Hydrogen sulphide production

Test isolates were inoculated to test tubes containing 5 ml of sterile H_2S medium and incubated at 28±2°C. The tubes were examined for H_2S production. The formation of black ring in the test tube was taken as positive for H_2S production.

3.3.5 Identification of general rhizobacterial isolates

The general bacterial isolates were identified upto generic level based on their morphology and biochemical characteristics as detailed for phosphate solubilizing bacteria in section 3.3.3.1 and 3.3.3.2 of this chapter.

3.4 Nitrogen fixation by *Azospirillum* isolates

The amount of nitrogen fixed in the broth culture by the free living N_2 fixing isolates was estimated by microkjeldahl method of Jackson (1973).

A loopful of 48 hours old culture of each isolate was inoculated to five ml N-free broth of respective medium and incubated for 48 hours. One ml of this broth was inoculated to 50 ml of respective N-free broth in 250 ml flasks. The flasks were incubated at 28±2°C for 15 days and 10 ml of this culture

was used for estimation of nitrogen. To the 10 ml of broth culture, five ml of concentrated H_2SO_4 and 200 mg catalyst mixture (potassium sulphate, copper sulphate and selenium in the ratio of 10:1:0.1) were added and allowed for digestion in a block digester for two hours to get the clear digest. The clear digest was cooled and diluted with distilled water upto 10 ml. This was distilled in a distillation unit after addition of 20 ml of 40 per cent sodium hydroxide solution to make the digest alkaline, in a Parnas-wayner type distillation unit. The evolved ammonia was absorbed in four per cent boric acid with mixed indicator and finally titrated with 0.05 N H_2SO_4 for colour change from red to green. From the volume of acid consumed, total nitrogen content was calculated and expressed as mg N per gram carbon source utilized.

3.5 P release by PSB strains

Isolates of phosphate solubilizing bacteria were inoculated to 100 ml Pikovskaya's broth (Pikovskaya, 1948) and incubated for two weeks at 28±2°C and the amount of Pi released in the broth (2 replications) was estimated in comparison with a set of uninoculated controls. The broth cultures were centrifuged at 9,000 rpm for 20 minutes in a Remi micro centrifuge to separate the supernatant from the cell growth and insoluble phosphate. Whenever, the supernatant was coloured due to pigmentation, about 1 g of activated charcol was added and

shaken until it become colorless and filtered through Whatman No. 1 filter paper. The available P content in the supernatant was estimated by phospomolybdic blue colour method (Jackson, 1973) as detailed below in 3.1.1.5.

3.5.1 Reagents used

Chloromolybdic acid

Chloromolybdic acid reagent was prepared by dissolving 7.5 g of ammonium molybdate in 150 ml distilled water to which 162 ml of concentrated HCl was added. The volume was made upto one litre with distilled water.

Chlorostannous acid

Chlorostannous acid reagent was prepared by dissolving 25 g of $SnCl_2$ $2H_2O$ in 100 ml concentrated HCl and making the volume to one litre with distilled water. Both the reagents were stored in amber coloured bottle in a refrigerator. One ml of the culture filtrate was taken in a 50 ml volume flask to which 10 ml of chloromolybdic acid was added and mixed thoroughly. The volume was made upto approximately three forth with distilled water and then 0.25 ml chlorostannous acid was added to it. Immediately the volume was made to 50 ml with distilled water. After 15 minutes, the blue colour developed was read on a spectrophotometer at 610 nm using a reagent blank. Simultaneously, a standard curve was prepared using various

concentrations of standard 2 ppm KH_2PO_4 solution. The amount of phosphorus solubilized was calculated from the standard curve.

3.6 Production of IAA and GA

The representative isolates of *Azospirillum*, phosphate solubilizers and general rhizobacteria isolated from soybean were tested for production of IAA and GA by paper chromatography following the method of Vancura and Macura (1960) as detailed below.

One ml of the 48 hours old cultures were inoculated to 50 ml of the respective broth media supplemented with L-tryptophan (sodium malate broth, Okon *et al.*, 1977, for *Azospirillum*; Pikovskaya's broth, Pikovskaya, 1948 for P-solubilizers and nutrient broth, Anon., 1957 for general rhizobacteria) and the flasks were incubated at 28±2°C for 20 days.

3.6.1 Extraction of growth regulators

The broth cultures after the incubation period were centrifuged at 4000 rpm for 40 minutes. The supernatant collected in EM flasks was acidified to pH 2.8 to 3.0 using 1 NHCl and extracted in 40 ml butanol in a 1000 ml separating funnel (Podjil and Seveik, 1960). The butanol extract was subjected to centrifugation at 5000 rpm for 10 minutes. The

organic layer was separated out from aqueous layer in a separating funnel. The organic layer was air-dried and the dried residue was dissolved in 0.5 ml of methanol.

3.6.2 Preparation of solvent mixture

Isopropanol (Analar grade), ammonia (0.85 sp gr) and double distilled water in the ratio of 10:1:1 by volume were transferred into a clean dry 500 ml capacity separating funnel and mixed thoroughly by various shaking for five minutes. The mixture was allowed to stand for five minutes to achieve a distinct separation of organic and aqueous phases. The aqueous phase was transferred into a clean sterile petriplate and kept in a chromatography chamber for saturation as a stationary phase. Whereas, organic phase was used as a mobile phase by pouring the solvent into the trough.

3.6.3 Preparation of indicator spray for detection of IAA and GA

Three ml of H_2SO_4 was diluted to 20 ml with double distilled water in a 100 ml volumetric flask to which 500 mg of ferric chloride was added and dissolved. The volume was made upto 100 ml with double distilled water and then used for spraying to detect IAA and GA produced by cultures.

3.6.4 Quantitative estimation of IAA and GA

3.6.4.1 Extraction

Bacterial cultures producing IAA and GA were inoculated to sterilized Czapek's solution (Mahadevan and Sridhar, 1984) supplemented with 0.005 M L-tryptophan. They were incubated at 37°C for 7 days in dark. After 7 days of incubation, the cultures were centrifuged at 6000 rpm to remove the bacterial cells and the supernatant was collected in a conical flask and used for estimation of IAA and GA.

3.6.4.2 IAA estimation

Twenty five ml of the supernatant was collected and the pH was adjusted to 2.8 using 1N HCl in a 100 ml conical flask. Equal volume of diethyl ether was added to it and incubated in dark for four hours. Extraction of IAA was done at 4°C in a separating funnel using diethyl ether. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, two ml of methanol was added, pooled and the IAA present in the methanol extract was determined using the method of Gorden and Paleg (1957). To 0.5 ml of methanol extract, 1.5 ml of distilled water and 4 ml Sapler's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% perchloric acid) was added and incubated in dark for one hour. The intensity of pink colour developed was read at 535 nm in a

spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity of IAA in the culture filtrate was determined and expressed as µg/25 ml of the medium.

3.6.4.3 GA estimation

Twenty five ml of the culture filtrate was taken in a test tube to which two ml of zinc acetate was added. After two minutes, two ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 minutes. To five ml of this supernatant, was added five ml of 30 per cent HCl and incubated at 20°C for 75 minutes. The blank sample was treated with five per cent HCl and the observance of the sample as well as blank was measured at 254 nm in a spectrophotometer. The amount of GA present in the extract was calculated from the standard curve and expressed as µg/25 ml of the medium. The standard curves of IAA and GA were prepared by using graded concentrations of IAA and GA_3.

3.7 Biocontrol test

Five efficient strains from each group of *Rhizobium*, *Azospirillum*, phosphate solubilizers and three efficient strains from general rhizobactera were studied for their potentiality of biocontrol. This biocontrol test was done against the pathogen *Sclerotium* collected from the Dept. of Plant Pathology.

The pathogen was spotted in the center marked area in the petriplate containing respective media and test isolate was spotted on the side of pathogen and the plate was kept for incubation for 3-4 days. Zone of inhibition indicates whether test isolate suppress the growth of pathogen or vice versa.

3.8 Evaluation of efficient strains under pot culture condition

A pot culture experiment was conducted to study combined effect of different efficient cultures, isolated from soybean rhizosphere, on the growth and nutrient uptake of soybean plants as detailed below.

3.8.1 Preparation of pots

The medium black soil collected from E block of Main Research Station, University of Agricultural Sciences, Dharwad was mixed thoroughly, sieved and filled in the earthen plots of 30 cms diameter at the rate of 18 kg per pot. The required quantity of FYM (90 gm pot^{-1}) was weighed separately for each pot and incorporated into the soil.

3.8.2 Soil characteristics

The pH of the soil was determined in 1:2.5 soil solution using a pH meter.

The soil was analysed for its available nitrogen by kjldahl method (Jackson, 1973) and organic carbon content by wet oxidation method (Jackson, 1973). The available phosphorus was determined by Olsen's method (Olsen *et al.*, 1954) and the available potassium by flame photometer method (Stanford and English, 1949). Similarly microbiological analysis of the soil was carried out by serial dilution and plate count.

3.8.3 Selection of efficient strain

One most effective strain of *Rhizobium*, *Azospirillum*, phosphate solubilizer and general rhizobacteria were selected based on their nodulation status, bio-controlling capacity, production of growth promoting substances, nitrogen fixation and P-solubilizing ability, for pot culture to see their effect either singly or in combination.

3.8.4 Fertilizers

The recommended dose of fertilizers for soybean was 40:80:25 kg NPK per hectare. N in the form of urea, P in the form of single superphosphate and K in the form of muriate of potash were applied to soil as basal dose at the time of sowing.

3.8.5 Seed inoculation and sowing

Soybean seeds of var. JS 335 collected from soybean improvement scheme at UAS, Dharwad, were used in the experiment. Healthy seeds with 85 per cent germination were sown in plots at five spots, to which one ml of the efficient inoculum broth was added according to the treatment schedule. One set of pots were kept as control without adding inoculum. After 10 days thinning was done to retain only three seedling in each pot.

3.8.6 Treatments

Four efficient strains selected namely *Rhizobium* (RhN_{23}), phosphate solubilizer ($PSBN_1$), *Azospirillum* ($AzoN_7$), general rhizobacteria (GBN_5) were compared with an uninoculated control. The performance of these strains was further evaluated in single application and in different combinations (Table 3).

3.9 Observations

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

Table 3. Treatment details of pot culture experiment.

Treatments

T_1	→ Control
T_2	→ *Rhizobium* (RhN_{23})
T_3	→ Phosphate solubilizer ($PSBN_1$)
T_4	→ *Azospirillum* ($AzoN_7$)
T_5	→ General rhizobacterial isolate (GBN_5)
T_6	→ *Rhizobium* + Phosphate solubilizer
T_7	→ *Rhizobium* + *Azospirillum*
T_8	→ *Rhizobium* + General rhizobacteria
T_9	→ Phosphate solubilizer + *Azospirillum*
T_{10}	→ Phosphate solubilizer + General rhizobacteria
T_{11}	→ *Azospirillum* + General rhizobacteria
T_{12}	→ *Rhizobium* + Phosphate solubilizer + *Azospirillum*
T_{13}	→ *Rhizobium* + Phosphate solubilizer + General rhizobacteria
T_{14}	→ *Rhizobium* + *Azospirillum* + General rhizobacteria
T_{15}	→ Phosphate solubilizer + *Azospirillum* + General rhizobacteria
T_{16}	→ *Rhizobium* + Phosphate solubilizer + *Azospirillum* + General rhizobacteria

1. **Plant height :** The average height of the three soybean plants was recorded at 30, 45 and 60 DAS and expressed in cm.

2. **Number of branches :** The average number of branches of three soybean plants were counted at 30, 45 and 60 DAS.

3. **Number of nodules per plant :** The Number of nodules on roots of each plant was counted and their mean was expressed as number of nodules per plant at 45 and 60 DAS.

4. **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 60 DAS and at harvest.

5. **Chlorophyll content :** Chlorophyll content of soybean plant was measured at 45 DAS and at 60 DAS.

Estimation of chlorophyll content

The total chlorophyll at 45 and 60 DAS 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 minutes. 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.

$$\text{Total chlorophyll} = 20.2\ (A\ 645) + 8.02\ (A\ 663) \times \frac{V}{100 \times w \times a}$$

(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)

6. **Grain yield :** Yield of soybean grains per plant was recorded at the time of harvest.

7. **Dry weight yields of both root and shoot :** The dry matter content of soybean plants was recorded at harvest. From uprooted plants, the root and shoot portions were separated and were separately air dried. The plant samples were oven dried at 60°C to constant weight. The shoot and root dry weights were recorded and expressed in g per plant.

8. **Nutrient uptake (N and P) :** The oven dried plant samples were ground to fine powder and used for estimation of nitrogen and phosphorous content.

3.9.1 Estimation of nitrogen

the total content in the plant sample was estimated following the microkjeldahl method as outlined by Jackson, (1973) The analysis was done with 500 mg of oven dried finely

ground samples which were digested with five ml of concentrated H_2SO_4 in presence of 200 mg catalyst mixture (containing potassium sulphate, copper sulphate and selenium in 100:10:1 ratio). The samples were digested on a microkjeldahl digestion unit till a clear solution was obtained. The digest was cooled and diluted with distilled water. The digested samples were distilled after adding 20 ml of 40 per cent NaOH to make the digest alkaline in a Parnar-wayers type semimicrokjeldahl distillation unit. The evolved ammonia was absorbed in four per cent boric acid solution and titrated against 0.05 NH_2SO_4. A standard was run by using 1 mg of nitrogen per five ml solution of ammonium sulphate and the titre values were converted to mg of nitrogen and per cent nitrogen was calculated.

3.9.2 Estimation of phosphorus

Five hundred mg of plant sample was taken in a 250 ml capacity conical flask and was added with 2.5 ml of concentrated HNO_3. The flask was swirled to moisten, the entire sample and then placed on a hot sand bath for 30 minutes and then on the electric hot plate at 180°C to 200°C. The suspension was boiled until taken nearly to dryness.

3.9.2.1 Wet oxidation

Five ml of tri acid mixture (Conc HNO_3, Conc H_2SO_4 and 60 per cent $HClO_4$ in the ratio of 10:1:4) was added to pre digested sample and further digestion was carried out at 180°C to 200°C on a digestion mantle until the residue in the flask became clear white. The content of the flask were collected and added with 10 to 15 ml of 6 N HCl and stirred well. The acid digest was transferred to 50 ml volumetric flask and the volume was made upto 50 ml with distilled water. From this wet oxidised digested color, 'P' was estimated by Vanadomolybdate phosphoric yellow colour method (Jackson, 1973).

Ten ml of wet oxidised digested sample was taken in a 50 ml volumetric flask and 10 ml of Vanadomolybdate reagent was added. The volume was made upto 50 ml with distilled water and allowed to react for 30 minutes. The yellow colour developed was read at 490 nm using spectrophotometer. The P-content was obtained by the standard curve.

For obtaining standard curve, 0.439 gm of KH_2PO_4 was dissolved in distilled water and made upto 1000 ml in a volumetric flask (100 ppm P-solution). Aliquots of 1 to 10 ml were transferred to 50 ml volumetric flask and 10 ml vanadomolybdate reagent was added to each flask including blank. The volume was made upto 50 ml with distilled water. The yellow colour developed was read after 10 minutes in a

spectronic-2-D soectrophotometer at 490 nm. The standard curve was obtained by plotting a graph as concentration along X-axis and corresponding observance along Y-axis.

3.10 Statistical analysis

The statistical analysis of the data in pot experiments were carried out as described by Panse and Sukhatme (1985) for completely randomized design as well as by Steel and Torrie (1960) for Dunkun's Multiple Range test.

Experimental Results

IV. EXPERIMENTAL RESULTS

Investigations were carried out on the growth promoting rhizobacteria of soybean for their abilities to fix atmospheric nitrogen, solubilize insoluble mineral phosphate, suppressing pathogens and production of plant growth promoting substances. Based on these characteristics, attempts were made to select most efficient strains and study their combined inoculation effect on growth and nutrient uptake of soybean under pot culture experiments. The results pertaining to present studies are interpreted in this chapter.

4.1 Studies on morphological and biochemical characteristics

4.1.1 *Rhizobium*

All the *Rhizobium* isolates were subjected to various morphological, cultural and biochemical characters and results pertaining to these studies are presented in Table 4. Morphologically, all the isolates exhibited typical bradyrhizobial colony characters. They produced smooth raised circular white to light pink coloured, small sticky colonies indicating that either they did not absorb or weakly absorbed the congored colour on YEMA + CR medium.

All the isolates formed well developed colonies on YEMA plates after seven days of incubation indicating that they were slow growers, except six isolates which exhibited fast growth. When these isolates were subjected for gram straining, it was found that they were gram negative. When these isolates are subjected for acid or alkali production test, it was noticed that only six isolates were acid producing rest of the isolates alkali producers. On glucose peptone agar and Hofer's alkali broth, six isolates exhibited very poor growth and the remaining isolates did not exhibit any growth. On ketalactose medium six isolates did not produced lactonic acid while the rest of them did not produce lactonic acid.

Nodulation ability of the bacterial isolates was tested using the soybean (*Glycine max* (L.) Merill) as test plant. Out of 30 isolates, 24 isolates were capable of nodulating on the test plant (Table 8). Therefore, only these cultures were retained for further studies.

4.1.2 *Azospirillum*

Fifteen isolates obtained on semi solid N-free malate medium were further characterized for their morphology and physiology (Table 5). All the strains showed pellicle formation in the semisolid N-free malate medium. All strains were gram negative having spiral shape with corkscrew motility.

Table 4. Morphological, physiological and biochemical characters of isolates collected.

Isolate No.	Appearance on YEMA	Rate of growth	Gram reaction	Growth on glucose peptone agar	Lactonic acid production test	Acid or Alkali Product -ion	Growth on Hofers alkali broth	Nodulation test
RhN1	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN2	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN3	Light Pink Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN4	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN5	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN6	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN7	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN8	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN9	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN10	Light pink, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN11	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN12	Creamy Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN13	White, Sticky	F	-ve	Faint growth	P	Acid	Yes	-ve
RhN14	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN15	White, Sticky	S	-ve	Faint growth	P	Alkali	Yes	-ve
RhN16	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN17	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN18	White, Sticky	F	-ve	Faint growth	P	Acid	Yes	-ve
RhN19	White creamy, Sticky	F	-ve	Faint growth	P	Acid	Yes	-ve
RhN20	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN21	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN22	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN23	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN24	White, Sticky	F	-ve	No growth	NP	Acid	No	+ve
RhN25	Light pink, sticky	F	-ve	Faint growth	P	Acid	Yes	-ve
RhN26	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN27	White, Sticky	F	-ve	Faint growth	P	Acid	Yes	-ve
RhN28	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN29	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve
RhN30	White, Sticky	S	-ve	No growth	NP	Alkali	No	+ve

S= Slow F= Fast NP= Not produced P= Produced

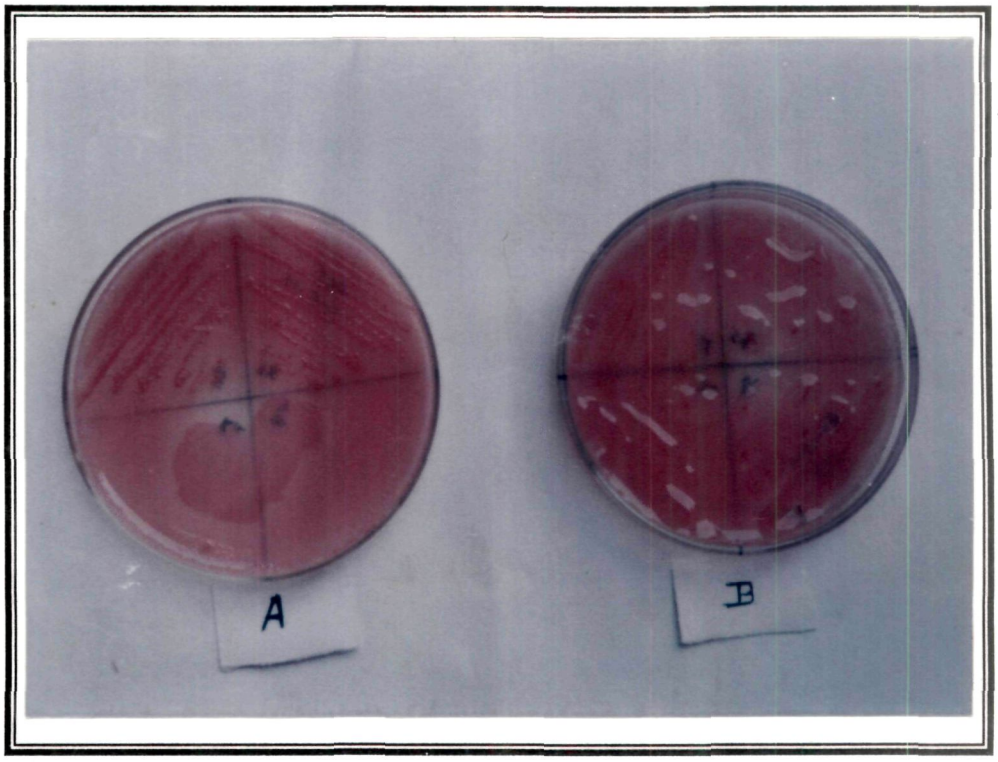

Plate 1a. Growth of *Rhizobium* on the petri plate containing YEMA media.

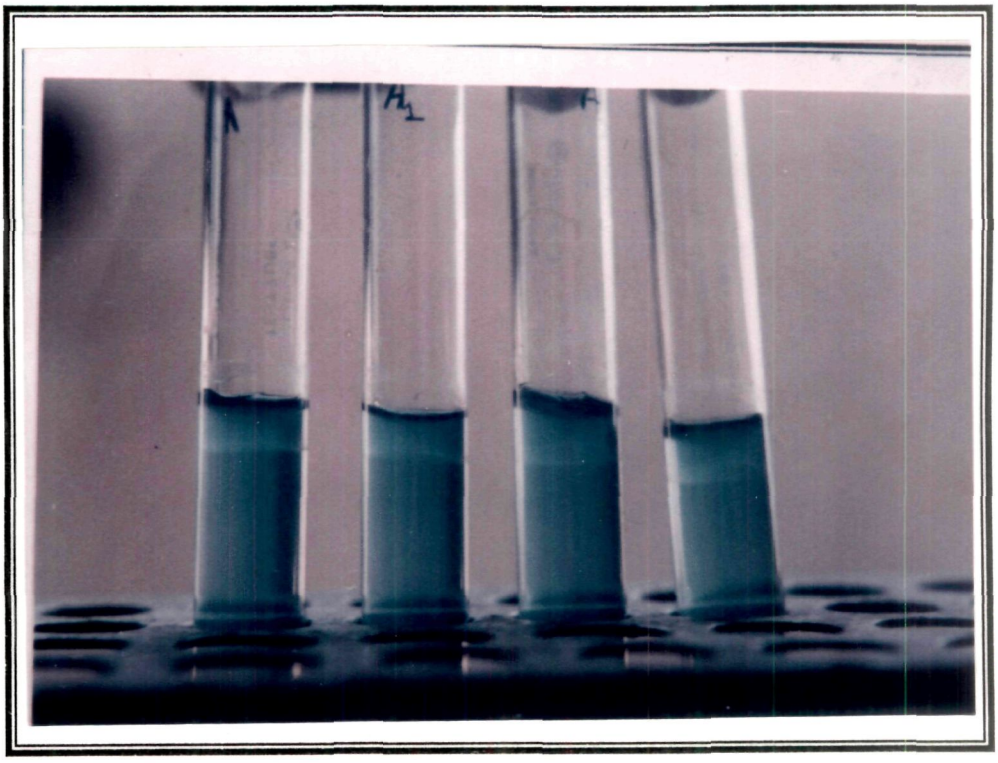

Plate 1b. Pellicle formation of *Azospirillum* isolates on semi solid N-free malate media.

About eight isolates showed pale white and smooth colonies, five isolates showed pink and curled colonies and two colonies showed brown, smooth to curled colonies, when grown on potato infusion agar. When grown on nutrient agar medium, it was found that 13 strains showing white to pale white, raised colonies whereas two strains showed white and smooth colonies.

When these isolates were subjected for carbon source utilization, sucrose supported medium growth for seven isolates, poor growth for six isolates and no growth of two strains. Dextrose supported medium growth for seven strains and poor growth for seven strains when as one strain showed no growth. Citrate and malate supported medium to better growth for almost all the strains.

4.1.3 Phosphte solubilizers

Based on morphological and biochemical tests 18 representative bacterial isolates which showed clear solubilizing zones on Pikovskaya's tricalcium phosphate medium were identified upto generic level. The results obtained are presented in Table 6.

All the 18 isolates were gram negative having rod shaped cells. Based on their morphological and biochemical characteristics, the isolates were found to be associated with

Table 5. Identification of *Azospirillum* isolates based on morphological and physiological characteristics.

Isolate No.	Colony morphology on N free Malate media	Pellicle formation	Cell shape	Gram reaction	Motility	Growth on Nutrient agar	Growth on Potato infusion agar	Carbon source utilization				Expected genus
								Sucrose	Dextrose	Malate	Citrate	
$AzoN_1$	Pale white shiny and small	Present	Spiral	-ve	Corkscrew	Pale white and raised	White and Smooth	MG	MG	MG	MG	*Azospirillum*
$AzoN_2$	Medium and wrinkled	Present	Spiral	-ve	Corkscrew	Pale white and raised	Pink and curled	MG	MG	MG	MG	*Azospirillum*
$AzoN_3$	White dense small	Present	Spiral	-ve	Corkscrew	White and raised	Pink and curled	PG	PG	MG	MG	*Azospirillum*
$AzoN_4$	Spindle and pale green colour	Present	Spiral	-ve	Corkscrew	White and raised	White and Smooth	MG	PG	MG	BG	*Azospirillum*
$AzoN_5$	Spindle and transparent	Present	Spiral	-ve	Corkscrew	Pale white and raised	Pale white & smooth	PG	MG	BG	SG	*Azospirillum*
$AzoN_6$	White dense small	Present	Spiral	-ve	Corkscrew	White and raised	Pink and curled	PG	PG	BG	BG	*Azospirillum*
$AzoN_7$	White dense and medium	Present	Spiral	-ve	Corkscrew	White and raised	Pink and curled	MG	MG	MG	MG	*Azospirillum*
$AzoN_8$	Small pale germ transparent	Present	Spiral	-ve	Corkscrew	White and raised	Pale white & smooth	PG	PG	BG	BG	*Azospirillum*
$AzoN_9$	Pale shiny white, small	Present	Spiral	-ve	Corkscrew	White and raised	Brown & smooth	PG	MG	MG	MG	*Azospirillum*
$AzoN_{10}$	White, dense small	Present	Spiral	-ve	Corkscrew	White and raised	Brown and curled	MG	MG	BG	BG	*Azospirillum*
$AzoN_{11}$	White, dense medium	Present	Spiral	-ve	Corkscrew	Pale white and raised	Pink and curled	MG	MG	BG	MG	*Azospirillum*
$AzoN_{12}$	Pale germ, medium and transparent	Present	Spiral	-ve	Corkscrew	Pale white & raised	Pale white & smooth	NG	PG	BG	BG	*Azospirillum*
$AzoN_{13}$	Pale green & transparent	Present	Spiral	-ve	Corkscrew	White and smooth	Pale white & smooth	NG	PG	BG	MG	*Azospirillum*
$AzoN_{14}$	Pale shiny white, medium	Present	Spiral	-ve	Corkscrew	Pale white and raised	Pale white & smooth	MG	NG	MG	BG	*Azospirillum*
$AzoN_{15}$	Pale white small raised	Present	Spiral	-ve	Corkscrew	White and smooth	White & Smooth	PG	PG	BG	MG	*Azospirillum*

PG = Poor growth MG = Medium growth BG = Better growth NG = No growth

the genera *Pseudomonas*, *Enterobacter* and *Xanthomonas*. Sixteen out of 18 isolates belonged to the genus *Pseudomonas*, one to *Enterobacter* and one to *Xanthomonas*.

4.1.4 General rhizobacteria

Eight major representative isolates from general rhizobacteria were characterized for their morphological and biochemical characteristics and results were presented in Table 7. All the isolates were gram negative having rod shaped cells. Based on morphological and biochemical tests out of eight isolates, six were found to be *Pseudomonas* and two were *Enterobacter* spp. The six isolates belonging to genus *Pseudomonas* showed negative reaction for starch hydrolysis acid and gas production and H_2S production test and showed positive for gelatin liquefaction (except one showing negative) and catalase activity. Whereas, two isolates belonging to genera *Enterobacter* showed positive for all biochemical test except H_2S production.

4.2 Nodulation test

Preliminary screening of 30 *Rhizobium* isolates conducted on soybean plant for nodulation test are presented in Table 8.

The number of nodules, formed by inoculation of different strains demonstrated a variation ranging from 3.0 to 14.0 nodules per plant. Significantly maximum number of nodules

Table 6. Identification of PSB isolate based on morphological and biochemical characteristics.

Isolate No.	Morphological characters			Biochemical test						
	Colony morphology	Gram react.	Cell shape	Starch hydrolysis	Gelatin liquefaction	Catalase activity	Acid produc-tion	Gas produc-tion	H_2S producti-on	Probable genus
$PSBN_1$	Small, white, circular & raised	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_2$	Large yellowish, circular and slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_3$	Medium, yellow, circular, slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	+ve	*Pseudomonas*
$PSBN_4$	Pale yellow, medium & raised	-ve	rod	-ve	-ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_5$	Large transparent and slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	+ve	*Pseudomonas*
$PSBN_6$	White, small & slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_7$	White, small & slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_8$	Small, white, circular raised	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_9$	Medium, circular and yellow	-ve	rod	+ve	-ve	+ve	+ve	+ve	-ve	*Enterobacter*
$PSBN_{10}$	Shiny white, round and slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_{11}$	Medium, shiny white, round & slimy	-ve	rod	+ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*

$PSBN_{12}$	Pale yellow, medium & raised	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_{13}$	Medium, shiny white, round & slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_{14}$	Medium, yellowish, slimy & round	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_{15}$	Small, white & round	-ve	rod	+ve	-ve	+ve	+ve	-ve	-ve	*Xanthomonas*
$PSBN_{16}$	Small, circular, transparent, white	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
$PSBN_{17}$	Small, circular white, slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	+ve	*Pseudomonas*
$PSBN_{18}$	Medium, yellow, circular & slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*

Table 7. Identification of the general rhizobacterial isolates based on morphological and biochemical characteristics.

Isolate No.	Morphological characters			Biochemical test						
	Colony morphology	Gram react.	Cell shape	Starch hydrolysis	Gelatin liquifaction	Catalase activity	Acid product -ion	Gas produc -tion	H_2S producti- on	Probable genus
GBN_1	Large with regular margin pale yellow & slimy	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
GBN_2	Big, white, circular & slimy	-ve	rod	-ve	-ve	+ve	-ve	-ve	-ve	*Pseudomonas*
GBN_3	Large with margin slimy pale yellow	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
GBN_4	Large, spreading yellow slimy & raised	-ve	rod	+ve	+ve	+ve	+ve	+ve	-ve	*Enterobacter*
GBN_5	Small yellow to white slimy & raised circular	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
GBN_6	Large spreading yellow slimy & raised	-ve	rod	+ve	+ve	+ve	+ve	+ve	-ve	*Enterobacter*
GBN_7	Small, smooth white circular & raised	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*
GBN_8	Small, circular, smooth white	-ve	rod	-ve	+ve	+ve	-ve	-ve	-ve	*Pseudomonas*

were recorded in the plants inoculated with isolate RhN_{23} (14.0) when compared with the others followed by RhN_5 (12.0), RhN_{30} (11.66) and RhN_3 (11.0).

However, six isolates were not capable of nodulating the plants. The nodule dry weight varied from 3.0 mg/plant to a maximum of 28.0 mg/plant. The maximum dry weight was recorded in isolate RhN_{23} (28.0 mg) and RhN_3 (28.0) followed by RhN_5 (27.5 mg), RhN_{11} (21.0 mg) and RhN_{30} (19.0 mg).

4.3 Nitrogen fixation by *Azospirillum*

The amount of nitrogen fixed by *Azospirillum* isolates are presented in Table 9. The results clearly indicate that all the isolates of *Azospirillum* were able to fix atmospheric nitrogen. The isolates showed 9.10 to 14.36 mg of N/g of glucose added. Significant differences in nitrogen fixation by the isolate was also observed. Among the *Azospirillum* isolates $AzoN_7$ showed highest amount of nitrogen fixation (14.36 mg), followed by $AzoN_{14}$ (13.56 mg), $AzoN_5$ (13.56), $AzoN_4$ (12.56 mg) and $AzoN_{10}$ (12.50 mg).

4.4 Phosphate solubilization

The data on the amount of tricalcium phosphate (TCP) solubilized in the broth by the PSB strains isolated from the different regions are presented in Table 10. The amount of P-released from the TCP by the phosphate solubilizing bacteria

Table 8. Performance of various *Rhizobium* isolates on root nodulation and nodule dry weight.

Rh Isolates	Nodule number (/plant)	Nodule dry weight (mg/plant)
RhN_1	6.33	7.5
RhN_2	5.33	6.0
RhN_3	11.0	28.0
RhN_4	3.33	7.0
RhN_5	12.0	27.5
RhN_6	4.0	3.0
RhN_7	8.0	12.0
RhN_8	7.33	13.5
RhN_9	3.0	6.0
RhN_{10}	7.0	12.0
RhN_{11}	7.0	21.0
RhN_{12}	7.0	13.0
RhN_{13}	NNF	NIL
RhN_{14}	8.0	9.5
RhN_{15}	NNF	NIL
RhN_{16}	10.0	16.5
RhN_{17}	4.0	6.5
RhN_{18}	NNF	NIL
RhN_{19}	NNF	NIL
RhN_{20}	9.0	12.5
RhN_{21}	11.0	12.5
RhN_{22}	9.0	6.5
RhN_{23}	14.0	28.0
RhN_{24}	4.0	3.5
RhN_{25}	NNF	NIL
RhN_{26}	5.66	5.5
RhN_{27}	NNF	NIL
RhN_{28}	9.0	12.0
RhN_{29}	6.0	11.0
RhN_{30}	11.66	19.0
SEm±	0.553	0.328
CD at 1%	1.647	0.977

NNF – Nodules not formed

Table 9. Nitrogen fixation by *Azospirillum* isolates.

Isolate No.	Amount of N_2-fixed in mg/gm of carbon source utilized
***Azospirillum* isolates**	
$AzoN_1$	9.10
$AzoN_2$	10.36
$AzoN_3$	12.31
$AzoN_4$	12.33
$AzoN_5$	12.56
$AzoN_6$	10.95
$AzoN_7$	14.36
$AzoN_8$	9.56
$AzoN_9$	11.35
$AzoN_{10}$	12.50
$AzoN_{11}$	12.00
$AzoN_{12}$	10.13
$AzoN_{13}$	11.65
$AzoN_{14}$	13.96
$AzoN_{15}$	9.60
Sem±	0.920
CD at 1%	2.741

Table 10. Phosphate solubilization by PSB isolates

Isolates	TCP solubilized (mg P/100 ml medium)
$PSBN_1$	47.5
$PSBN_2$	26.1
$PSBN_3$	39.0
$PSBN_4$	38.5
$PSBN_5$	37.0
$PSBN_6$	33.5
$PSBN_7$	24.0
$PSBN_8$	37.5
$PSBN_9$	34.0
$PSBN_{10}$	43.0
$PSBN_{11}$	22.0
$PSBN_{12}$	19.0
$PSBN_{13}$	36.0
$PSBN_{14}$	47.5
$PSBN_{15}$	25.5
$PSBN_{16}$	40.5
$PSBN_{17}$	33.05
$PSBN_{18}$	33.1
SEM +	0.391
CD at 1%	1.165

ranged from 19 to 47.5 mg/100 ml broth. Among the PSB isolates, $PSBN_1$ released highest amount of P (47.5 mg/100 ml broth) and was significantly superior over rest of the P-solubilizing bacteria. Among the remaining $PSBN_{10}$ isolate released about 43 mg/100 ml broth followed by $PSBN_{16}$ (40.5 mg/100 ml broth), $PSBN_3$ (39.0 mg/100 ml broth) and PSB (38.5 mg/100 ml broth).

4.5 Production of IAA and GA

The representative isolates of phosphate solubilizers, *Azospirillum* and general rhizobacteria were examined for production of Indole Acetic Acid (IAA) and Gibberellic Acid (GA) by qualitative and quantitative methods and the results are presented in Table 11. The amount of IAA produced by different strains of phosphate solubilizers ranged from 5.4 µg to 24.0 µg/25 ml broth, whereas, the amount of GA produced ranged from 1.21 µg to 9.28 µg/25 ml broth. All the strains of phosphate solubilizers are capable of producing both IAA and GA. The strain $PSBN_4$ was found to produce the highest amount of IAA (24.0 µg/25 ml) followed by $PSBN_1$ (22.0 µg/25 ml). The least producer was $PSBN_5$ (5.4 µg/25 ml).

The amount of GA produced by the PSB strains varied significantly and ranged from 1.21 µg to 9.28 µg/25 ml broth. $PSBN_{11}$ produced the highest amount of GA (9.28 µg/25 ml)

followed by $PSBN_6$ (7.41 µg/25 ml), $PSBN_{16}$ (5.88 µg/25 ml) and $PSBN_1$ (5.80 µg/25 ml). The lowest production of GA was in $PSBN_{14}$ (1.21 µg/25 ml).

In case of *Azospirillum*, all the strains are capable of producing IAA and three strains are not capable of producing GA. The amount of IAA produced ranged from 5.3 µg to 36.20 µg/25 ml. The strain $AzoN_7$ was found to produce significantly highest amount of IAA (36.20 µg/25 ml) than others followed by $AzoN_8$ (31.86 µg/25 ml), $AzoN_{14}$ (30.55 µg/25 ml) and $AzoN_4$ (28.70 µg/25 ml). The least producer was $AzoN_2$ (5.3 µg/25 ml). The amount of GA produced ranged from 1.21 µg to 4.75 µg/25 ml. The significant highest producer was $AzoN_7$ (4.75 µg/25 ml) than others followed by $AzoN_{10}$ (4.20 µg/25 ml). Three strains i.e., $AzoN_4$, $AzoN_5$ and $AzoN_{13}$ were not capable of producing GA.

In case of general rhizobacteria except two strains, all were capable of producing IAA and GA. GBN_4 was not producing IAA and GBN_8 was not providing GA. The amount of IAA production range from 7.5 µg to 21.10 µg/25 ml. The significant producer was GBN_5 (21.10 µg/25 ml) followed by GBN_6 (18.1 µg/25 ml).

The amount of GA produced ranged from 0.60 µg to 3.38 µg/25 ml. The significant highest amount of GA was produced

Table 11. Production of IAA and GA by PSB, *Azospirillum* and General rhizobacterial isolates.

Isolate	Amount of IAA produced (µg/25 ml)	Amount of GA produced (µg/25 ml)
PSB isolates		
$PSBN_1$	22.0	5.85
$PSBN_2$	7.5	1.28
$PSBN_3$	10.5	4.55
$PSBN_4$	24.0	2.66
$PSBN_5$	5.4	2.19
$PSBN_6$	10.5	7.41
$PSBN_7$	18.75	1.71
$PSBN_8$	17.00	3.31
$PSBN_9$	18.00	2.90
$PSBN_{10}$	7.4	1.98
$PSBN_{11}$	9.3	9.28
$PSBN_{12}$	6.8	3.44
$PSBN_{13}$	11.5	3.46
$PSBN_{14}$	8.4	1.21
$PSBN_{15}$	18.1	2.60
$PSBN_{16}$	10.5	5.88
$PSBN_{17}$	12.8	3.66
$PSBN_{18}$	18.67	2.17
***Azospirillum* isolates**		
$AzoN_1$	9.30	1.21
$AzoN_2$	5.3	1.27
$AzoN_3$	9.16	3.44
$AzoN_4$	28.70	-
$AzoN_5$	17.95	-
$AzoN_6$	17.90	1.23
$AzoN_7$	36.20	4.75
$AzoN_8$	31.86	2.51
$AzoN_9$	11.30	3.66
$AzoN_{10}$	15.51	4.20
$AzoN_{11}$	9.50	1.34
$AzoN_{12}$	28.30	-
$AzoN_{13}$	28.50	1.33
$AzoN_{14}$	30.95	1.27
$AzoN_{15}$	13.90	3.50
General rhizobacterial isolates		
GBN_1	7.5	2.98
GBN_2	10.5	1.84
GBN_3	11.5	0.80
GBN_4	-	0.60
GBN_5	21.10	3.38
GBN_6	18.1	1.65
GBN_7	8.4	1.65
GBN_8	10.4	-
SEm±	0.186	0.022
CD at 1%	0.554	0.065

by GBN_5 (3.38 µg/25 ml) when compound with others followed GBN_1 (2.98 µg/25 ml).

4.6 Biocontrol test

To know the efficiency of selected efficient strains against the pathogens i.e., *Sclerotium* bio-control test was conducted and results obtained were presented in Table 12. Among the *Rhizobium* strains only two strains showed positive effect *viz.*, RhN_{23} and RhN_{30}. Among PSB isolates only one strain i.e., PSB N_1 was showed positive effect. Among general rhizobacterial strains, only one strain GBN_5 expressed positive effect. Whereas, in case of *Azospirillum*, all the tested isolates showed negative effect.

4.7 Effect of PGPR on plant parameters

4.7.1 Plant height

Significant differences in the plant height of soybean observed at 30, 45 and 60 DAS (Days After Sowing) due to various inoculation treatments presented in Table 13.

At 30 DAS, T_{12} (*Rhizobium* + PSB + *Azospirillum*) recorded significantly higher plant height (25.4 cm) which was on par with T_{14} (*Rhizobium* + *Azospirillum* + General rhizobacteria), T_6 (*Rhizobium* +P), T_{16} (*Rhizobium* +P+A+G) and T_{13} (R+P+G) which were 25.33, 25.26, 24.83 and 24.50 cm, respectively.

Table 12. Potentiality of PGPR as bio-control agents for the control of *Sclerotium*.

	Strains	Bio-control Effect
Rhizobium Strains	RhN_3	-ve
	RhN_5	-ve
	RhN_{16}	-ve
	RhN_{23}	+ve
	RhN_{30}	+ve
PSB Strains	$PSBN_1$	+ve
	$PSBN_4$	-ve
	$PSBN_{10}$	-ve
	$PSBN_{11}$	-ve
	$PSBN_{16}$	-ve
Azospirillum strains	$AzoN_4$	-ve
	$AzoN_5$	-ve
	$AzoN_7$	-ve
	$AzoN_{10}$	-ve
	$AzoN_{14}$	-ve
General rhizobacterial strains	GBN_5	+ve
	GBN_6	-ve
	GBN_1	-ve

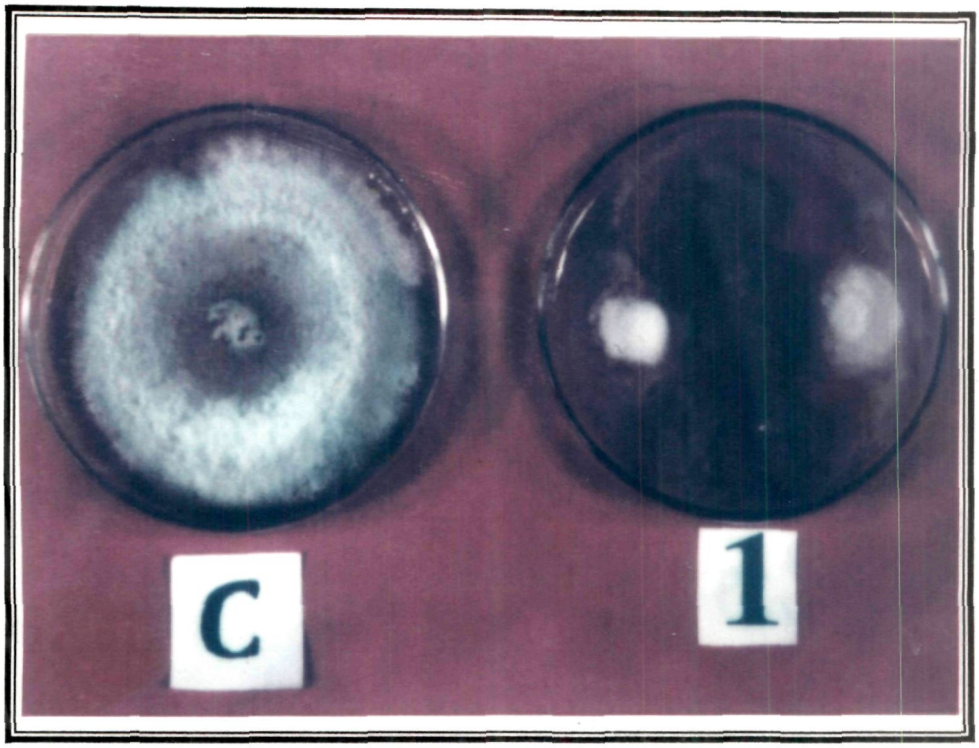

Plate 2a. Biocontrol test.

Plate 2b. Soybean plants grown under pot culture condition.

Among single inoculation treatments T_2 (*Rhizobium* alone) recorded maximum plant height and lowest plant height was with T_5 (General rhizobatceria alone) which was on par with control (T_1). Among dual inoculation treatments, T_6 (R+P) recorded significantly higher plant height as compared to other dual inoculated treatments. Among triple inoculation treatments, T_{12} (R+P+A) recorded significantly higher plant height (25.40 cm). However, it was on par with T_{13} (24.50 cm) and T_{14} (25.33 cm). Significantly lower plant height was recorded with T_{15} (21.73 cm).

At 45 DAS, T_{12} recorded significantly higher plant height (29.06 cm) when compared with rest of the treatments which was on par with T_2 (28.66 cm) and T_{16} (27.86 cm). Among single and dual inoculation treatments similar trend was followed as noticed in 30 DAS, but among triple inoculation treatments T_{12} showed significant higher plant height than others.

At 60 DAS, T_{12} recorded significantly higher plant height (35.53 cm) than others, which was on par with T_6 (35.30 cm), T_{14} (35.03) and T_{16} (35.30 cm).

In general, T_2, T_6 and T_{12} recorded highest plant height among single, dual and triple inoculations, respectively and these treatments including T_{16} are significantly superior over the control.

4.7.2 Number of branches per plant

Significant difference in the number of branches of soybean was noticed at 30, 45 and 60 DAS due to various inoculation treatments and were presented in Table 13.

At 30 DAS, more number of branches were recorded in inoculation treatments receiving T_6 (*Rhizobium* + PSB), T_{12} (Rhizoboium + PSB + *Azospirillum*), T_{13} (*Rhizobium* + PSB + General rhizobacteria) and T_{16} (*Rhizobium* + PSB + *Azospirillum* + general rhizobacteria) which were on par with T_7 (*Rhizobium* + *Azospirillum*) having 3.66 and significantly superior than rest of the treatments and control.

Among single inoculation T_5 (general rhizobacteria alone) showed lowest number of branches (2.0). Among dual inoculation T_6 (4.00) showed significant more number of branches when compared with rest of the treatments, which was on par with T_7 (3.66).

Among triple inoculation treatments T_{12} (4.0) and T_{13} (4.0) showed significant more number of branches than rest of the treatments.

At 45 DAS, inoculation treatments T_6, T_{12}, T_{13} and T_{16} showed more number of branches when compared with other treatments and significantly superior over single inoculated treatments and control.

Among single inoculation treatments T_2 (4.66) recorded more number of branches however, it was on par with rest of treatments. All single inoculation treatments were showed significantly more number of branches than control. Among the dual inoculation treatments T_6 (5.66) recorded more number of branches, which was on par with T_7 (5.00) and T_8 (5.00) and significantly superior over rest of treatments. Among triple inoculation treatments T_{12} (5.66) and T_{13} (5.66) recorded more number of branches and on par with other treatments.

At 60 DAS, significantly more number of branches were noticed/recorded in T_{12} (7.66) when compared with rest of the treatments and control. However, which was on par with T_6 (7.0), T_{13} (7.0) and T_{16} (7.33).

Among single inoculation treatments except T_5 (5.00) all treatments showed significantly more number of branches than control (4.67), T_2 showed maximum number of branches. In case of dual inoculation treatments T_6 (7.0) showed significantly more number of branches than other treatments except T_7 (6.33). Whereas, in case of triple inoculation treatments, significantly more number of branches were recorded in T_{12} (7.66) than other treatments which was on par with T_{13} (7.00).

In general T_{12}, T_{16} and T_2 showed more number of branches among triple, dual and single inoculations,

Table 13. Effect of inoculation of PGPR on plant height and number of branches.

Treatments	Plant height (cm) at			Number of branches		
	30 DAS	45 DAS	60 DAS	30 DAS	45 DAS	60 DAS
T_1 Control	19.267	22.933	28.900	2.00	3.66	4.67
T_2 R	23.467	26.267	34.333	3.00	4.66	6.00
T_3 P	21.567	24.800	32.167	3.00	4.33	5.66
T_4 A	21.133	24.167	31.400	3.00	4.33	5.66
T_5 G	19.767	23.167	28.967	2.00	4.00	5.00
T_6 R+P	25.267	28.667	35.300	4.00	5.66	7.00
T_7 R+A	23.500	26.600	34.400	3.66	5.00	6.33
T_8 R+G	23.200	26.433	33.967	3.00	5.00	6.00
T_9 P+A	21.733	25.667	33.067	3.00	4.33	5.66
T_{10} P+G	21.400	24.700	31.900	3.00	4.33	5.66
T_{11} A+G	21.200	23.667	31.533	3.00	4.33	5.66
T_{12} R+P+A	25.400	29.067	35.533	4.00	5.66	7.66
T_{13} R+P+G	24.500	27.133	35.033	4.00	5.66	7.00
T_{14} R+A+G	25.333	26.233	34.367	3.33	5.00	6.33
T_{15} P+A+G	21.733	25.500	32.867	3.00	5.00	5.66
T_{16} (R+P+A+G)	24.833	27.867	35.200	4.00	5.66	7.33
SEm±	0.459	0.584	0.370	0.118	0.276	0.276
CD at 1%	1.367	1.74	1.10	0.351	0.822	0.822

R= *Rhizobium* P= Phosphate solubilizer A= *Azospirillum* G= General Rhizobacteria

LEGEND

T_1 → Control

T_2 → *Rhizobium* alone RhN_{23} (R)

T_3 → Phosphate solubilizer alone $PSBN_1$ (P)

T_4 → *Azospirillum* alone $AzoN_7$ (A)

T_5 → General rhizobacterial isolate GBN_5 (G)

T_6 → *Rhizobium* + Phosphate solubilizer

T_7 → *Rhizobium* + *Azospirillum*

T_8 → *Rhizobium* + General rhizobacteria

T_9 → Phosphate solubilizer + *Azospirillum*

T_{10} → Phosphate solubilizer + General rhizobacteria

T_{11} → *Azospirillum* + General rhizobacteria

T_{12} → *Rhizobium* + Phosphate solubilizer + *Azospirillum*

T_{13} → *Rhizobium* + Phosphate solubilizer + General rhizobacteria

T_{14} → *Rhizobium* + *Azospirillum* + General rhizobacteria

T_{15} → Phosphate solubilizer + *Azospirillum* + General rhizobacteria

T_{16} → *Rhizobium* + Phosphate solubilizer + *Azospirillum* + General rhizobacteria

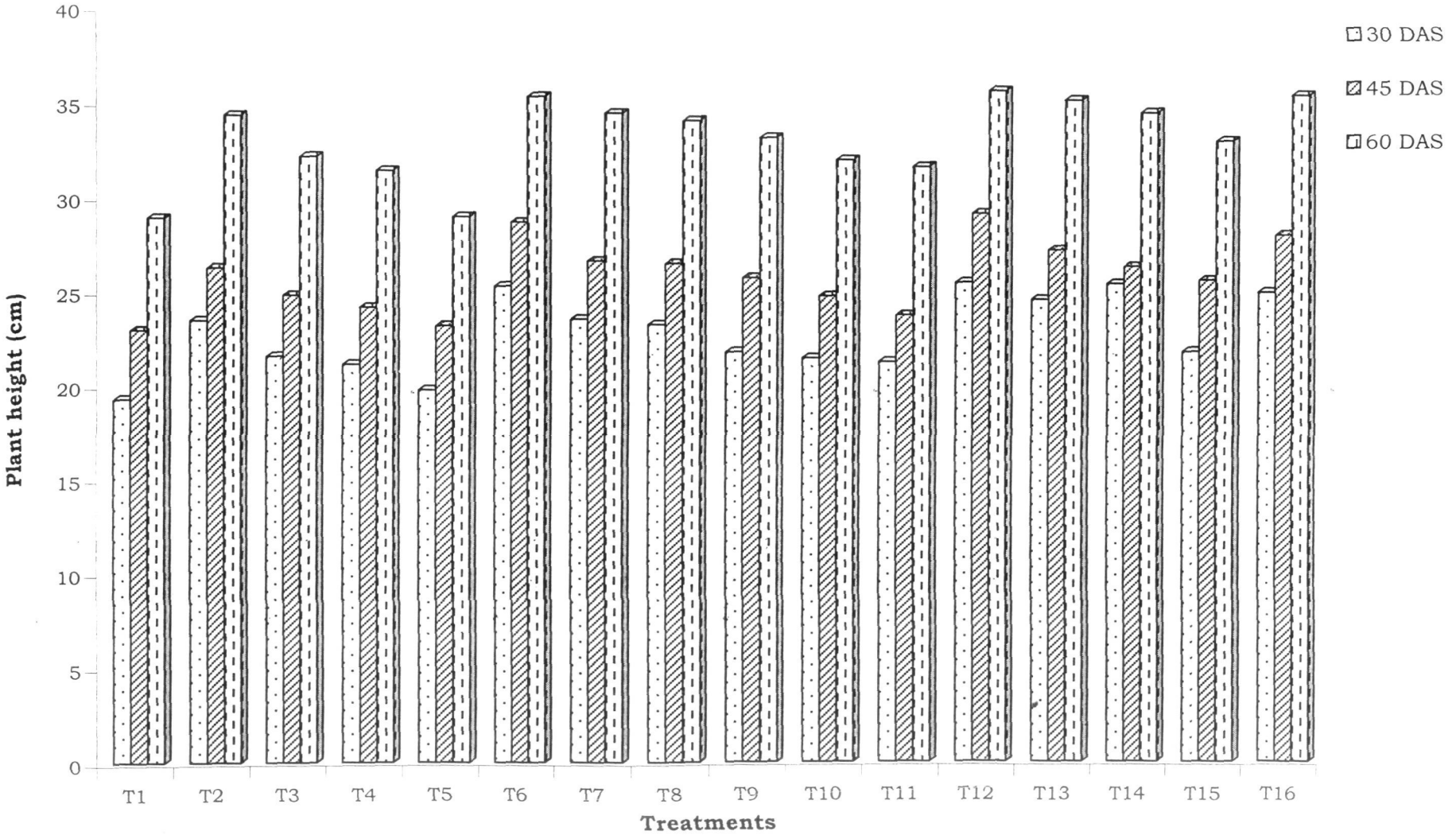

Fig. 1 Effect of inoculation of PGPR on plant height of soybean

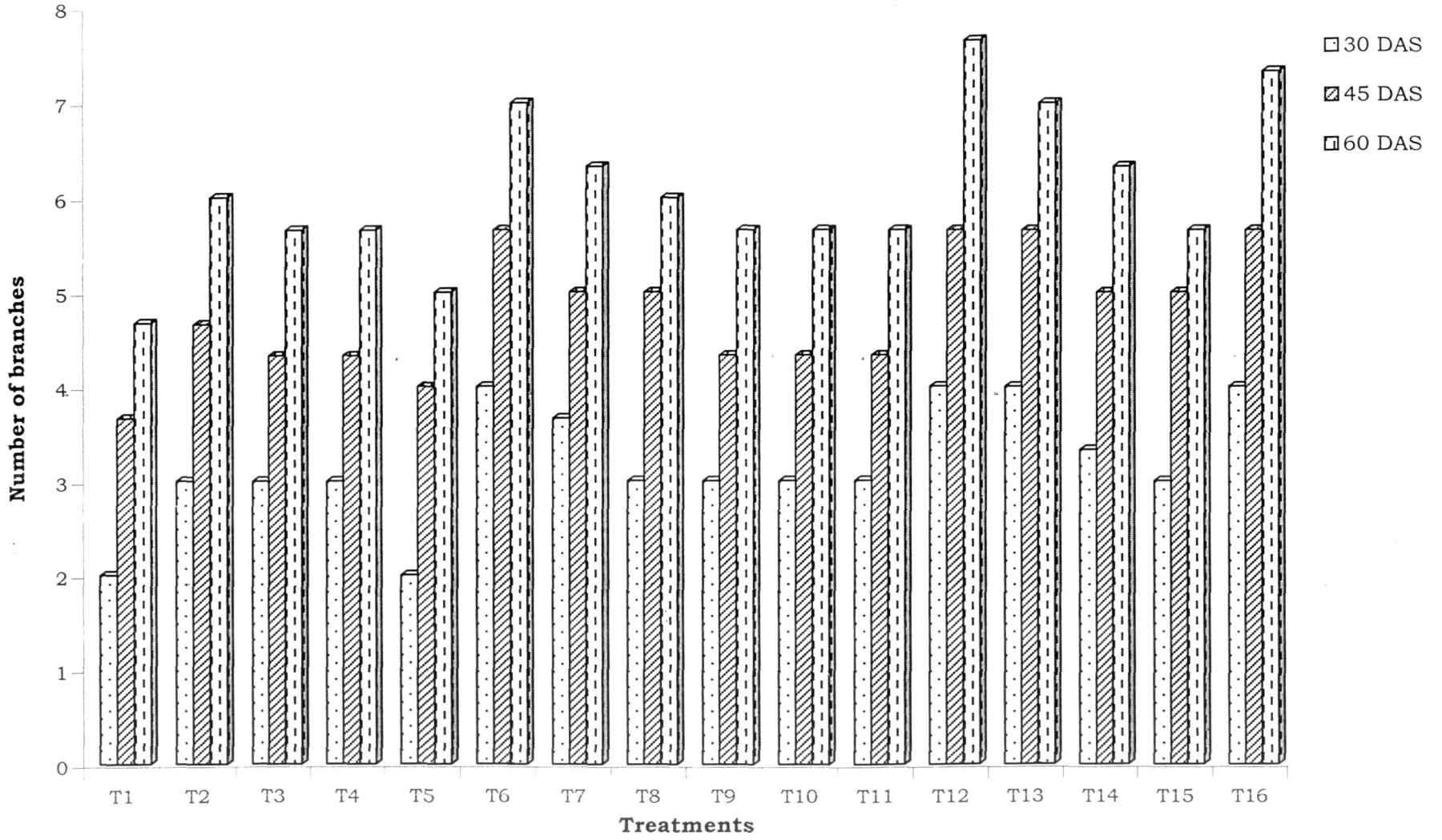

Fig. 2. Effect of inoculation of PGPR on number of branches of soybean.

LEGEND

T_1 → Control

T_2 → *Rhizobium* alone

T_6 → *Rhizobium* + Phosphate solubilizer

T_7 → *Rhizobium* + *Azospirillum*

T_8 → *Rhizobium* + General rhizobacteria

T_1 → Control

T_2 → *Rhizobium* alone

T_6 → *Rhizobium* + Phosphate solubilizer

T_{12} → *Rhizobium* + Phosphate solubilizer + *Azospirillum*

T_{16} → *Rhizobium* + Phosphate solubilizer + *Azospirillum* + General rhizobacteria

Plate 3a. Effect of inoculation of PGPR strains on growth of plant.

Comparing single and dual inoculations with control.

Plate 3b. Effect of inoculation of PGPR strains on growth of plant.

Comparing the treatments receiving single, dual, triple and combination of four organisms with control.

respectively and these inoculation treatments were significantly superior over control.

4.7.3 Nodule number

Variations in nodule number at 45 and 60 DAS as a consequence of different microbial inoculation and their interaction are presented in Table 14.

At 45 DAS, significantly more number of nodules per plant was recorded in T_{12} (32.00/plant) when compared with all other treatments and control and significantly lowest nodule number per plant was recorded in control (3.00/plant).

Among single inoculation treatments T_2 (21.00/plant) showed significantly superior over rest of the treatments. Among the dual inoculation treatments T_6 (30.0/plant) recorded significantly more nodule number per plant than rest of the treatments. In case of triple inoculation treatments T_{12} (32.0/plant) recorded significantly highest nodule number per plant when compared with rest of the treatments.

At 60 DAS, significantly more number of nodules per plant was recorded in T_{12} (36.00/plant) when compared with all other inoculation treatments and control except T_6 (35.00/plant) and T_{16} (35.66/plant) which were on par with T_{12}. Among single, dual and triple inoculation treatments similar pattern of results observed as recorded at 45 DAS.

The inoculation treatments involving *Rhizobium* showed more number of nodules. In general T_2, T_6, T_{12} and T_{16} showed more number of nodules among single, dual, triple and in the treatment involving four organisms, respectively.

4.7.4 Chlorophyll content

Chlorophyll content of soybean was significantly differed at 45 and 60 DAS due to various inoculation treatments and were presented in Table 14.

At 45 DAS, T_{12} (2.65 mg/g) showed significantly maximum chlorophyll content when compared with rest of the inoculation treatments and control, which was on par with T_{16} (2.48 mg/g). Among the single inoculation treatments T_2 (1.95 mg/g) showed more chlorophyll content than other treatments and all single inoculated treatments were significantly superior over control.

Among dual inoculation treatments, T_6 (2.43 mg/g) recorded maximum chlorophyll content, which was closely on par with T_7 (2.37 mg/g) and significantly superior over rest of the treatments. Significantly maximum chlorophyll content was noticed in T_{12} (2.65 mg/g) over the rest of the treatments in case of triple inoculation.

Similar trend in results were also observed at 60 DAS.

Table 14. Effect of inoculation of PGPR on number of nodules and chlorophyll content.

Treatments		Number of nodules/plant		Chlorophyll content (mg/g fresh weight)	
		45 DAS	60 DAS	45 DAS	60 DAS
T_1	Control	3.00	3.66	1.58	2.16
T_2	R	21.00	26.00	1.95	2.60
T_3	P	5.00	5.00	1.92	2.53
T_4	A	6.00	7.33	1.91	2.56
T_5	G	5.00	6.33	1.80	2.52
T_6	R+P	30.00	34.00	2.43	3.03
T_7	R+A	26.00	28.66	2.37	2.95
T_8	R+G	25.00	28.00	2.15	2.72
T_9	P+A	6.00	8.00	2.20	2.83
T_{10}	P+G	5.00	7.00	1.99	2.60
T_{11}	A+G	4.33	5.00	1.98	2.55
T_{12}	R+P+A	32.00	36.00	2.65	3.24
T_{13}	R+P+G	29.00	31.00	2.46	3.11
T_{14}	R+A+G	27.00	29.00	2.38	2.96
T_{15}	P+A+G	5.00	6.00	2.21	2.87
T_{16}	(R+P+A+G)	29.00	35.66	2.48	3.20
SEm±		0.479	0.373	0.060	0.028
CD at 1%		1.427	1.111	0.178	0.083

R= *Rhizobium* P= Phosphate solubilizer A= *Azospirillum* G= General Rhizobacteria

LEGEND

T_1 → Control

T_2 → *Rhizobium* alone RhN_{23} (R)

T_3 → Phosphate solubilizer alone $PSBN_1$ (P)

T_4 → *Azospirillum* alone $AzoN_7$ (A)

T_5 → General rhizobacterial isolate GBN_5 (G)

T_6 → *Rhizobium* + Phosphate solubilizer

T_7 → *Rhizobium* + *Azospirillum*

T_8 → *Rhizobium* + General rhizobacteria

T_9 → Phosphate solubilizer + *Azospirillum*

T_{10} → Phosphate solubilizer + General rhizobacteria

T_{11} → *Azospirillum* + General rhizobacteria

T_{12} → *Rhizobium* + Phosphate solubilizer + *Azospirillum*

T_{13} → *Rhizobium* + Phosphate solubilizer + General rhizobacteria

T_{14} → *Rhizobium* + *Azospirillum* + General rhizobacteria

T_{15} → Phosphate solubilizer + *Azospirillum* + General rhizobacteria

T_{16} → *Rhizobium* + Phosphate solubilizer + *Azospirillum* + General rhizobacteria

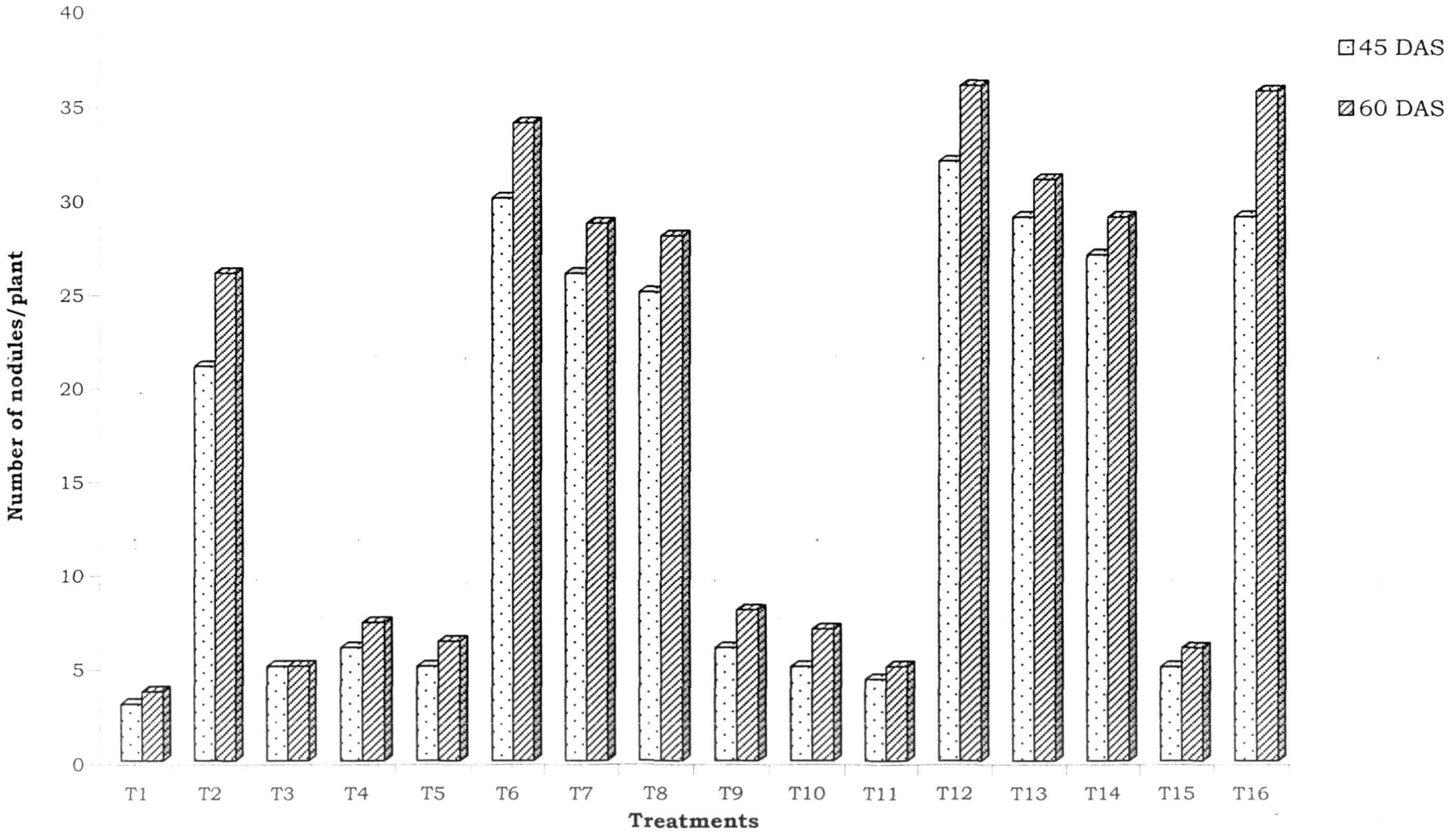

Fig. 3. Effect of inoculation of PGPR on number of nodules of soybean.

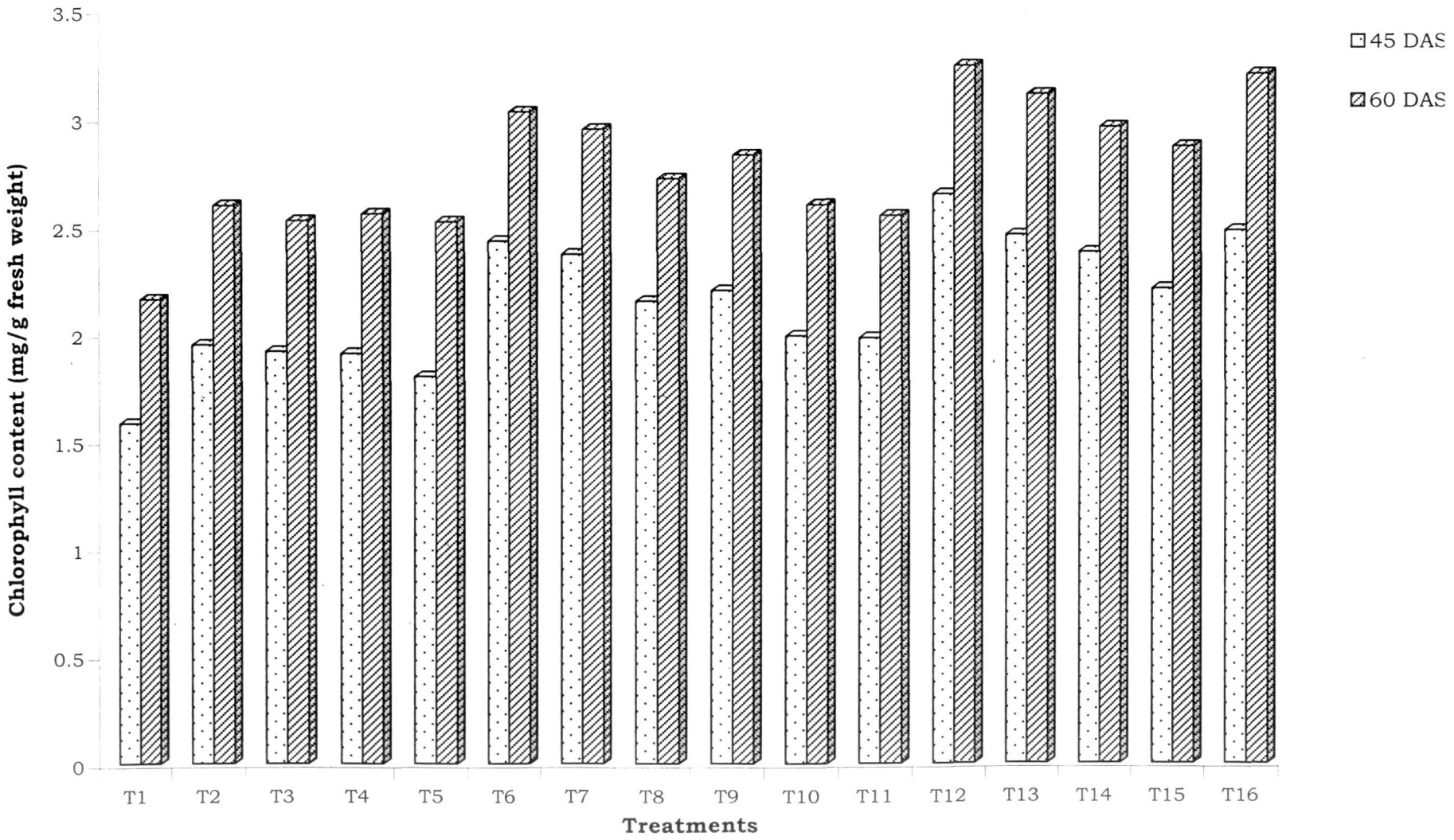

Fig. 4. Effect of inoculation of PGPR on chlorophyll content of soybean.

LEGEND

T_1	Control
T_2	*Rhizobium* alone
T_6	*Rhizobium* + Phosphate solubilizer

T_1	Control
T_{12}	*Rhizobium* + Phosphate solubilizer + *Azospirillum*
T_{16}	*Rhizobium* + Phosphate solubilizer + *Azospirillum* + General rhizobacteria

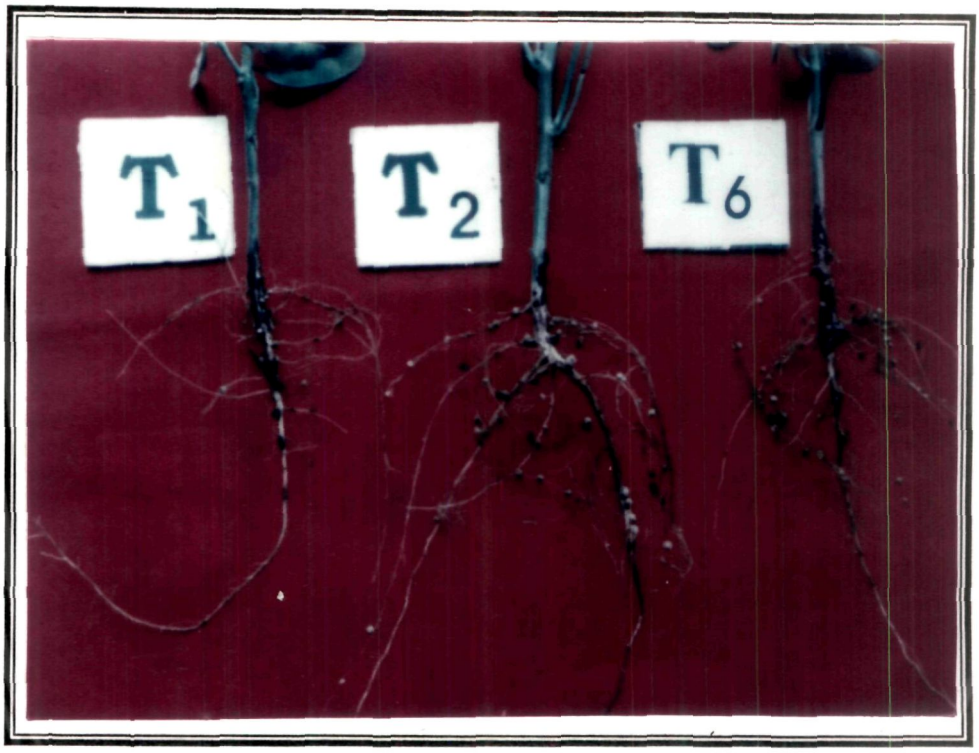

Plate 4a. Effect of PGPR strains on number of nodules.
Comparing the treatment receiving single and dual inoculations.

Plate 4b. Effect of PGPR strains on number of nodules.
Comparing the treatment receiving triple and inoculation of four organism.

4.7.5 Dry weight of shoots

The shoot dry weight of soybean plants was found to increase significantly at harvest due to inoculation of combined PGPR inoculants (Table 15).

The inoculation treatment T_{12} (6.148 g/plant) recorded significantly highest shoot dry weight when compared with rest of the inoculation treatments and control. It was followed by T_{16} which recorded 6.026 g. Lowest shoot dry weight was observed in control (4.102 g/plant).

Among the single inoculation treatments, T_2 (5.187 g/plant) recorded significantly highest shoot dry weight than other treatments and lowest shoot dry weight was recorded in T_5 (4.450 g/plant). Among the dual inoculation treatments, significantly highest shoot dry weight was recorded in T_7 (5.936 g/plant) when compared with rest of the treatments and lowest shoot dry weight was recorded in T_{10} (5.055 g/plant). In case of triple inoculation, T_{12} recorded significantly highest shoot dry weight than other treatments.

4.7.6 Dry weight of roots

All the inoculation treatments significantly enhanced the root dry weight of soybean plants over control (Table 15). Significantly highest root dry weight was noticed in the inoculation treatment receiving T_{12} (1.636 g) when compared

Table 15. Effect of inoculation of PGPR on dry weight yield at harvest.

Treatments		Shoot dry weight (g/plant)	Root dry weight (g/plant)
T_1	Control	4.102	0.987
T_2	R	5.187	1.384
T_3	P	5.022	1.350
T_4	A	5.049	1.336
T_5	G	4.450	1.186
T_6	R+P	5.749	1.525
T_7	R+A	5.936	1.592
T_8	R+G	5.189	1.389
T_9	P+A	5.436	1.476
T_{10}	P+G	5.028	1.351
T_{11}	A+G	5.055	1.340
T_{12}	R+P+A	6.148	1.636
T_{13}	R+P+G	5.753	1.537
T_{14}	R+A+G	5.941	1.591
T_{15}	P+A+G	5.443	1.479
T_{16}	(R+P+A+G)	6.026	1.633
SEm±		0.021	0.009
CD at 1%		0.062	0.026

R= *Rhizobium*
A= *Azospirillum*
P= Phosphate solubilizer
G= General Rhizobacteria

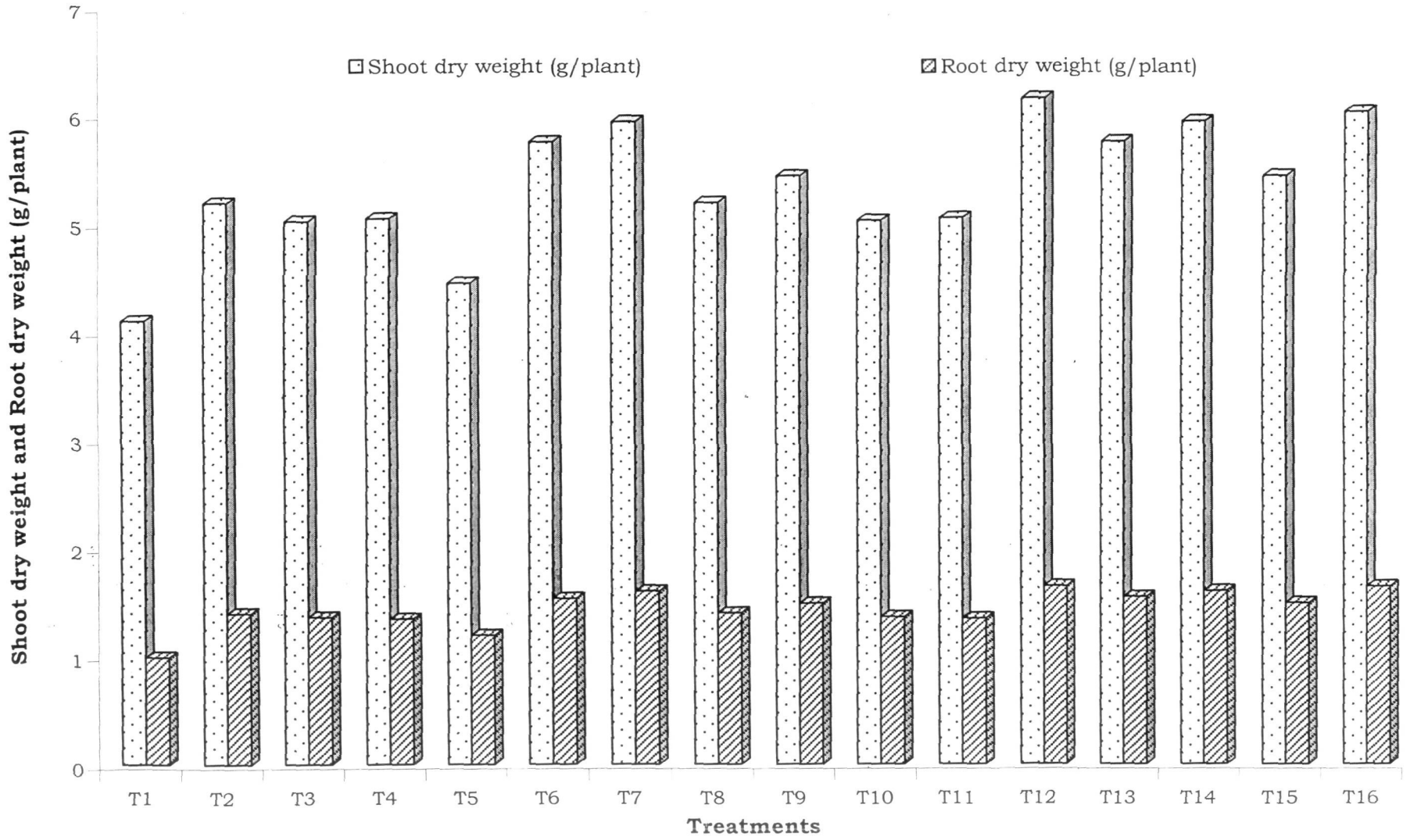

Fig. 5. Effect of inoculation of PGPR on shoot dry weight and root dry weight of soybean.

with rest of the treatments, however, which was closely on par with T_{16} (1.633 g).

Among the single inoculated treatments, T_2 (1.384 g) showed highest root dry weight which was significantly superior over rest of the treatments. Inoculation treatment receiving T_7 showed significant highest root dry weight when compared with rest of the treatments among the dual inoculation treatments. Whereas, in case of triple inoculated treatments T_{12} recorded significantly highest root dry weight than other treatments and lower root dry weight was noticed in T_{15} (5.443g).

4.7.7 Number of pods

Number of pods per plant was significantly increased due to various combined inoculation treatments at 60 DAS and at harvest (Table 16).

At 60 DAS, T_{16} showed significantly maximum number of pods per plant than rest of the treatments and control.

Among single inoculation treatments, T_2 (10.33) recorded more number of pods per plant followed by T_3 (9.66) and was significantly superior over rest of the treatments. Among dual inoculation treatments, significantly more number of pods per plant was recorded in T_6 (13.66) and T_7 (13.00) when compared with other treatments and T_{10} showed lowest number of pods per plant.

Among triple inoculation treatments, T_{13} (13.33) recorded more number of pods per plant and it was on par with rest of the treatments. Number of pods per plant recorded at harvest clearly showed that treatments receiving T_6 (23.66), T_{12} (24.66), T_{13} (23.66) and T_{16} (24.33) were significantly high and superior as compared to rest of the inoculation treatments and control, but the difference between them was non-significant.

Among single inoculation treatments, T_3 (19.00) recorded more number of pods per plant and was significantly superior over rest of the treatments except T_2 (18.33). Among dual inoculation treatments, T_6 (23.66) recorded significantly more number of pods per plant than other treatments. Among triple inoculation treatments, T_{12} and T_{13} recorded significantly more number of pods per plant when compared with rest of the treatments.

Among the highest results recorded from the treatments, T_{12} has showed good results when compare to T_2 and T_6 of single and dual inoculation, respectively.

4.7.8 Grain yield

Significant variations in grain yield of soybean plant due to inoculation of different microbial treatments was furnished in Table 16.

Significantly highest grain yield was noticed in T_{12} (12.25 g) when compared with all other inoculation treatments and control. However, which was on par with T_{16} (12.07 g). Lowest grain yield was recorded in control (7.43 g).

Among single inoculation, higher grain yield was recorded in T_2 (9.70 g) followed by T_4 (9.43 g) and T_3 (9.33 g) and these three treatments were significantly superior over T_5 (7.80 g) which was on par with control. Among dual inoculations, T_7 (11.48 g) recorded significantly higher grain yield than rest of the treatments. However, which was on par with T_6 (11.42 g) and T_9 (11.35 g). Among triple inoculations, significant highest yield was recorded in T_{12} when compared with rest of the treatments and lowest yield was recorded in T_{15} (11.37 g).

4.7.9.1 Shoot N content

The nitrogen content in the shoot of soybean plants was significantly increased due to inoculation of combined microbial inoculants as compared to control (Table 17).

Treatment inoculation receiving T_{16} (2.98%) was significantly superior over rest of the treatments and control (1.84%). However, which was on par with T_{14} (*Rhizobium* + *Azospirillum* + General bacteria) recorded 2.91 per cent.

Among the single inoculation treatments, T_2 (2.33%) showed higher N content followed by T_4 (2.27%). However, both

Table 16. Effect of inoculation of PGPR on number of pods and grain yield.

Treatments		Number of pods/plant		Grain yield (g/plant) at harvest
		60 DAS	At harvest	
T_1	Control	6.33	13.33	7.43
T_2	R	10.33	18.33	9.70
T_3	P	9.66	19.00	9.33
T_4	A	9.00	17.00	9.43
T_5	G	7.33	15.33	7.80
T_6	R+P	13.66	23.66	11.42
T_7	R+A	13.00	21.33	11.48
T_8	R+G	10.33	18.00	9.63
T_9	P+A	12.66	21.00	11.35
T_{10}	P+G	9.66	17.33	9.36
T_{11}	A+G	10.33	16.66	9.40
T_{12}	R+P+A	12.66	24.66	12.25
T_{13}	R+P+G	13.33	23.66	11.41
T_{14}	R+A+G	13.00	21.66	11.58
T_{15}	P+A+G	12.66	21.33	11.37
T_{16}	(R+P+A+G)	14.66	24.33	12.07
SEm±		0.300		0.200
CD at 1%		0.894		0.596

R= *Rhizobium* P= Phosphate solubilizer A= *Azospirillum* G= General Rhizobacteria

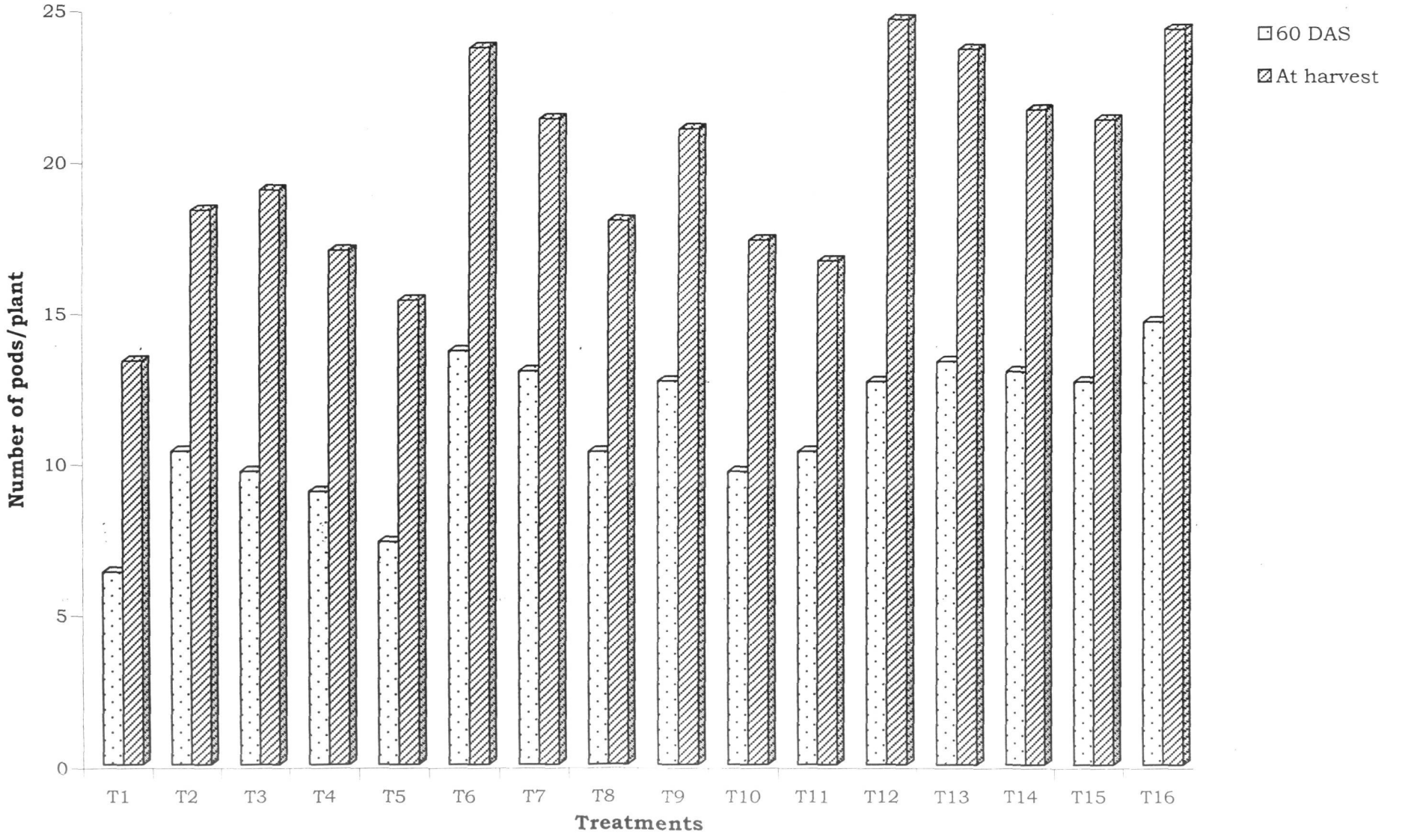

Fig. 6. Effect of inoculation of PGPR on number of pods of soybean.

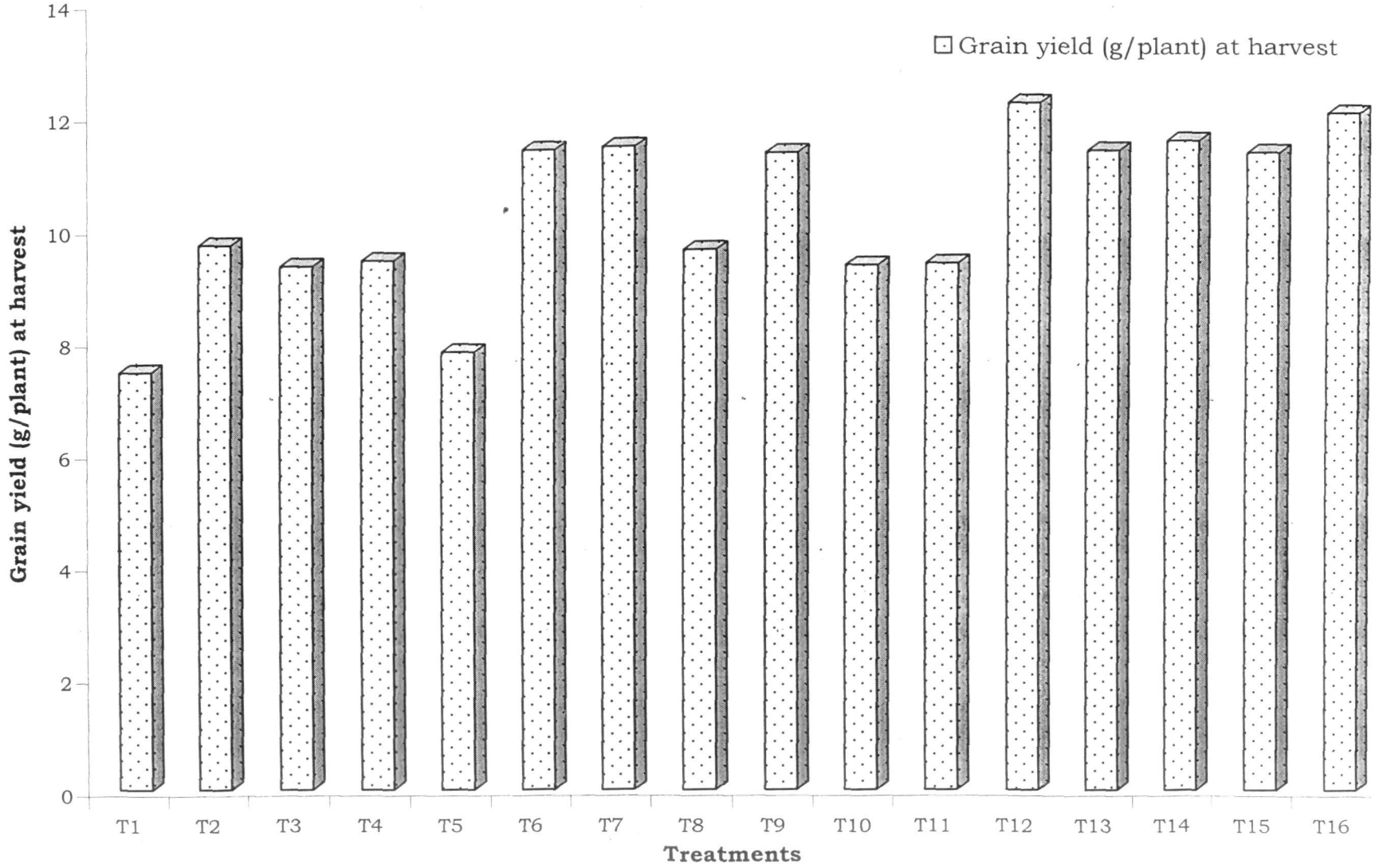

Fig. 7. Effect of inoculation of PGPR on grain yield of soybean.

are significantly superior over rest of the treatments. Among dual inoculation treatments, T_7 (2.80%) recorded significant higher N content when compared with other treatments and lowest N content was recorded in T_{10} (2.18%). Among triple inoculation treatments T_{14} (2.91%) showed significant higher N content than others,

4.7.9.2 Shoot N uptake

The nitrogen uptake in the shoot of soybean plant was found to be significantly higher in all the inoculation treatments over control (71 mg) T_{16} (171 mg) recorded significant highest N uptake when compared with the other treatments. Among single inoculation treatments, T_2 (117 mg) recorded significant highest N uptake in shoot than other treatments and lowest N uptake was recorded in T_5 (90 mg).

Among dual inoculation treatments, T_7 (160 mg) recorded significant highest N uptake in shoot when compared with rest of the treatments and lowest was recorded in T_{10} (107 mg). Among triple inoculation treatments, T_4 (166 mg) showed significant highest N uptake in shoot than other treatments and lowest was recorded in T_{15} (141 mg).

Table 17. Effect of inoculation of PGPR on N content and N-uptake

Treatments		Shoot	
		N content (%)	N-uptake (mg/plant)
T_1	Control	1.84	71
T_2	R	2.33	117
T_3	P	2.23	104
T_4	A	2.27	112
T_5	G	2.07	90
T_6	R+P	2.56	143
T_7	R+A	2.80	160
T_8	R+G	2.41	121
T_9	P+A	2.33	123
T_{10}	P+G	2.18	107
T_{11}	A+G	2.29	113
T_{12}	R+P+A	2.77	163
T_{13}	R+P+G	2.67	149
T_{14}	R+A+G	2.91	166
T_{15}	P+A+G	2.66	141
T_{16}	(R+P+A+G)	2.98	171
SEm±		0.027	0.565
CD at 1%		0.080	1.683

R= *Rhizobium* P= Phosphate solubilizer
A= *Azospirillum* G= General Rhizobacteria

4.7.9.3 Shoot P content

The phosphorus content in the shoot of soybean plants was significantly increased due to inoculation of various microbial inoculants (Table 18).

Treatment inoculation receiving T_{16} (0.42%) showed significant higher P content when compared with rest of the treatments.

Among the single inoculation treatments, T_3 (PSB alone) showed significant higher P content in the shoot than other treatments which recorded 0.30 per cent and lowest P content was recorded in T_4 (0.21%).

Among the dual inoculation treatments, T_{10} (PSB + General rhizobacteria) showed significant highest P content which recorded 0.38 per cent and lowest P content was recorded in T_7 (0.23%).

Among the triple inoculation treatments, T_{15} (0.39%) showed significant higher P content than rest of the treatments. However, which was on par with T_{13} (0.38%).

4.7.9.4 Shoot P uptake

The phosphorus uptake in the shoot of soybean plants was found to be significantly higher in all the inoculation treatments over control (6.64 mg).

Table 18. Effect of inoculation of PGPR on P content and P uptake in shoot at harvest.

Treatments		P content (%) in shoot	P uptake (mg/plant) in shoot
T_1	Control	0.17	6.64
T_2	R	0.22	11.14
T_3	P	0.30	14.78
T_4	A	0.21	10.37
T_5	G	0.25	10.88
T_6	R+P	0.33	18.50
T_7	R+A	0.23	13.17
T_8	R+G	0.26	13.07
T_9	P+A	0.30	15.94
T_{10}	P+G	0.38	18.73
T_{11}	A+G,	0.26	12.89
T_{12}	R+P+A	0.32	18.84
T_{13}	R+P+G	0.38	21.32
T_{14}	R+A+G	0.35	20.07
T_{15}	P+A+G	0.39	20.94
T_{16}	(R+P+A+G)	0.42	24.13
SEm±		0.005	0.313
CD at 1%		0.014	0.932

R= *Rhizobium* P= Phosphate solubilizer
A= *Azospirillum* G= General Rhizobacteria

Significant highest P uptake was recorded in T_{16} (24.13 mg) when compared with the rest of the treatments.

Among single inoculation treatments, T_3 (14.78 mg) recorded significant highest P uptake when compared with other treatments and lowest was recorded in T_4 (10.37 mg). Among dual inoculation treatments, T_{10} (18.73 mg) recorded significant higher P uptake than rest of the treatments. However, which was on par with T_6 (18.50 mg). Among triple inoculation treatments, T_{13} (21.32 mg) recorded higher P uptake followed by T_{15} (20.94 mg). However, both are significantly superior over rest of the treatments.

Discussion

V. DISCUSSION

The rhizosphere is a complex system consisting of the soil immediately adjacent to the roots, the root surface with its overlying slimecoat and the endorhizosphere. The rhizosphere microbial activity is influenced by root activities such as the exudation of organic substrates such as aminoacids, sugars, enzymes, carbohydrates, vitamins and several other chemicals. These chemical substances as well as microbial interactions that help in nutrient availability may have a profound influence on component of the soil microflora and thereby could have major effects on the growth and development of plants (Linderman, 1986). Hence, the crop productivity can be improved by manipulating the beneficial rhizosphere microorganisms. The effective microorganisms (EM) which means to indicate specific mixed cultures of known beneficial microorganisms may have an added advantage in the degraded agro-ecosystems of tropical countries. To maximize the beneficial plant growth responses, optimal combination of selected microbes is to be used, therefore it is important to identify the effective strains of beneficial microorganisms for the planting situation, based on their compatibility and combined efficacy, both *in vitro* and *in vivo* and employ this consortia of microorganisms in real agricultural situations for efficient management and production to promote plant growth

and yield. Contrary to the use of single inoculants, combined inoculation of two or more beneficial and compatible organisms have been shown to work better which indicates that in mixed cultures there is a better interaction of the introduced compatible organisms (Alagawadi and Gaur, 1988; Poi *et al.*, 1989; Patil *et al.*, 1992; Belimov *et al.*, 1995). Combined inoculations are said to work better than single inoculations based on the principle that greater the diversity and number of inhabitants, the higher the order of interaction and more stable the ecosystem (Higa and Wididana, 1991) and also they exert moiré accent effect (Patil *et al.*, 1992).

Although, the results of field tests have demonstrated the considerable potential of using combined inoculation of microorganisms to promote plant growth but the consistency of results is lacking. It can be because of the underlying mechanisms accounting for the phenomenon are not well understood. In case of growth benefit by dual inoculation, it is assumed that each beneficial microbe contributes something toward enhanced plant growth, such as increased nitrogen or soluble phosphate. The mechanisms may be much more complex that, and elucidating them should be subjected to future research. Furthermore, some of the bacteria involved may be interacting on more than one metabolic level i.e., P-solubilizers may also be auxin producers and N_2-fixers may

also solubilize phosphorus. It is well-known that certain soil properties-such as moisture holding capacity, pH, texture and organic matter content favour the establishment and activity of certain organisms. Therefore, effective use of dual or multiple inoculants will depend on matching the microorganisms of interest with the environment that favours its activities or altering the environment to favour the microorganisms. In addition, the ability of inoculated microbes to compete with the native flora and the influence of usual agronomic practices like tillage, fertilizer and pesticide application will also influence the outcome of inoculation.

In this context an attempt was made to know the effects of plant growth promoting rhizobacteria like *Rhizobium*, *Azospirillum*, phosphate solubilizers and general rhizobacteria on growth, nutrient uptake and yields of soybean. The results obtained pertaining to this study are discussed here under.

5.1 Isolation and characterization of PGPRs as N-fixers

5.1.1 *Rhizobium*

All the 24 cultures, isolated from the root nodules of soybean from different locations, were confirmed as *Rhizobium*. These isolates were gram negative short rods, slow growing bacteria, which formed white to creamy, raised circular sticky colonies on YEMA with congored. They exhibited poor growth on

glucose peptone agar and absence of growth in Holfer's alkaline broth. They did not produce 3-ketogalactose in ketolactose medium. These observations are in accordance with *Rhizobium* characteristics as described by Vincent (1970).

Out of 30 isolates, six isolates were slimy and absorbed colour of congored in YEMA plates. These isolates exhibited fair growth on ketalactose medium by producing 3-ketogalactase forming yellow ring around the colonies indicating these cultures were of *Agrobacterium* type, a common contaminant of root nodule bacteria as reported by Bernaertz and Deley (1963). Growth of these six isolates in Holfer's alkaline broth once again illustrates that these were possible contaminants like *Agrobacterium* which tolerate a pH of 11 in the media whereas, *Rhizobium* can not tolerate high pH as reported by Hofer (1935).

No growth or poor growth of *Rhizobium* was observed on glucose peptone agar plates and this is in accordance with report of Stowers and Elkan (1980).

The rate of growth of 24 isolates on YEMA was slow and these isolates took almost seven days to form a visible colony. This character is also considered to authenticate *Bradyrhizobium* isolates since *B. japonium* is slow grower. This slow growth was once gain confirmed by production of alkali metabolites. On YEMA + BTB medium these slow growers produced alkali metabolites which changed the colour of BTB

(pH indicator) from green to blue (neutral to alkaline) whereas, fast growers produced acidic metabolites changing the colour from green to yellow. These results were consistent with the report of Bromfield and Jones (1980) who reported that many fast growing rhizobia produce acid from sugar when grown in culture medium.

The ability to nodulate on suitable host plant is considered as an important characteristic for any isolate to confirm their identity as rhizobia (Vincent, 1970; Bergerson *et al.*, 1971).

In the present study 24 isolates nodulated on soybean (*Glycine max*) host plant in about 21 days of inoculation. This result confirmed the isolates as rhizobia. Inability of the remaining six isolates to nodulate in soybean plant once again illustrated that they were not *Rhizobium* possibly some contaminants from soil saprophytic forms. Hence, these isolates were excluded for further study.

5.1.1.1 Efficiency test of isolates or nodulation test

Among the 24 isolates, RhN_{23} formed more number of nodules which was statistically significant over other isolates. It was followed by isolate numbers RhN_5, RhN_{30}, RhN_3, RhN_{21} and RhN_{16}. The variability among the strains for their capacity to nodulate and to fix atmospheric nitrogen have been reported

(Mal and Yadav, 1972; Sundar Rao, 1974). Number of nodules formed due to inoculation of strains was between 3.0 to 14.0.

In the present investigation, maximum nodule dry weight was found in plant inoculated with isolate RhN_{23} (28.09 mg). The dry weight nodules recorded by isolates RhN_{23}, RhN_3, RhN_5, RhN_{11} and RhN_{30} was significantly higher than other isolates. Nodule dry weight recorded by different isolates did not show any relation with nodule number. This is in agreement with the results of Schiffmann and Label (1973). The results of the glass house studied so for clearly indicated that isolate RhN_{23}, RhN_3, RhN_5 and RhN_{30} performed well with regard to their efficiency in nodulation.

5.1.2 *Azospirillum*

Based on cell morphology i.e., curved and twisted shape, characteristic rotating corkscrew type of motility and formation of subsurface pellicle in the nitrogen free semisolid malate medium, the associative diazotorphic isolates from surface sterilized soybean roots were identified as *Azospirillum* as suggested by Kerig (1976). The amount of nitrogen fixed by the *Azospirillum* isolates ranged from 9.10 to 14.36 mg/g of carbon source utilized. Isolation of *Azospirillum* from the roots of numerous cereals, legumes and cultivated grasses from tropical, subtropical and temperate soils world wide have been reported (Okon *et al.*, 1977; Agarwal and Tilak, 1988;

Jaskowska, 1995). Almost similar range of nitrogen fixation as that recorded in the present study have also been noted earlier (Dobereiner and Boddey, 1980; Prathibha, 1993; Tamilvendan and Purushothaman, 1996 and Maheshkumar, 1997).

5.1.3 Phosphate solubilizers

Out of 18 isolates identified based on morphological and biochemical characteristics, 16 belonged to the genus *Pseudomonas*, one to the genus *Enterobacter* and remaining one to the genus *Xanthomonas* indicating the dominance of *Pseudomonas* sp. having P-solubilizing ability occurrence of phosphate solubilizing bacteria in the rhizosphere of various crop plants has been well documented (Gaur, 1990). Reports on solubilization of insoluble phosphates by microorganisms have been made by several workers (Gaur *et al.*, 1973; Bardiya and Gaur, 1972; Wani and Patil, 1979 and Wani, 1980). The amount of tricalcium phosphate (TCP) solubilized by the 18 bacterial isolates ranged from 19 to 47.5 mg/100 ml broth.

5.1.4 General rhizobacteria

About eight general bacteria isolated from soybean rhizosphere were purified and subjected to identification. Out of eight predominant bacterial isolates, six belonged to the genus *Pseudomonas* and remaining two to the genus *Enterobacter*.

These results are in agreement with the earlier findings of several workers (Rovira and Davey, 1977; Curl and Truelove, 1986; Lambert *et al.*, 1987; Chan *et al.*, 1994 and Maheshkumar, 1997) who also observed predominant occurrence of *Pseudomonas*, *Xanthomonas*, *Bacillus* and *Enterobacter* in the rhizosphere.

5.2 Production of IAA and GA

The isolated organisms were also examined for another property i.e., production of plant growth promoting substances such as IAA and GA. Production of IAA and GA by phosphate solubilizers, *Azospirillum* and general rhizobacteria were reported by many workers (Barea *et al.*, 1976; Reynders and Vlassak, 1979; Tien *et al.*, 1979; Sattar and Gaur, 1987) provides support for the present investigation.

All the isolates of phosphate solubilizers are able to produce IAA and GA. In case of *Azospirillum* all the isolates produced IAA whereas three isolates are unable to produce GA. Whereas, in case of general rhizobacteral isolates except two strains all are able to produce both IAA and GA. These results are in confirmity with the earlier findings with respect to IAA and GA production by *Azospirillum*, Phosphate solubilizers and General rhizobacteria (Omay *et al.*, 1992; Bar and Okon, 1993, Sattar and Gaur, 1987 and Leinhos and Vacek, 1993).

5.3 Biocontrol efficiency test

Only the efficient promoting rhizobacterial isolates are subjected for biocontrol test. Among them two strains of *Rhizobium*, two strains of *Azospirillum* and one strain of phosphate solubilizers suppressed the growth of *Sclerotium* pathogen whereas general rhizobacteria did not show any pathogen supression. These results are in conformity with the earlier findings with respect to microorganisms as biocontrolers (Siddiqui and Mahamood, 1995, Wakimoto, 1987; Gallardo *et al.*, 1989). The suppression of pathogen may be either due to the production of antibiotics substances or production of biocides. In view of their ability to control pathogenic flora the PGP rhizobacteria is also referred as Plant Health Promoting Rhizobacteria (PHPR) as it improves soil quality.

5.4 Effect of plant parameters

5.4.1 Plant height and number of branches

The plant height and number of branches of soybean cultivars JS-335 increased significantly with the multiple inoculation when compared with single inoculations and control at 30, 45 and 60 days of growth. The maximum plant height and number of branches was recorded in the treatment 12, which received combined inoculation of *Rhizobium*, *Azospirillum* and phosphate solubilizer, which was however on par with

treatment 16, which received combined inoculation of *Rhizobium*, *Azospirillum*, phosphate solubilizer and general rhizobacteria. However, increase in crop growth due to single inoculation of beneficial organisms like *Azospirillum, Rhizobium* and phosphate solubilizers (*Pseudomonas*) over control has been reported by several workers (Wani *et al.*, 1978; Defreitas *et al.*, 1997, Upadhyay *et al.*, 1999; Sharma *et al.*, 1999) in various crops.

But, the combined inoculation of two or more beneficial organisms have been reported to perform better than the single inoculation treatments (Patil et al., 1992; EL-Mokadem *et al.*, 1989; Balachandar and Nagarajan, 1999; Alagawadi and Gaur, 1988, 1992 and Balmurugan and Gunasekaran, 1996). The increase in plant growth in the combined inoculation of treatment of all the three organisms may be ascribed for enhanced N and P nutrient uptake (Rao and Dhir, 1993). However, another mechanism with which it is possible to explain similar effects, is the synthesis and exudation of plant growth promoting substances like IAA and GA (Tien *et al.*, 1979). IAA and GA are known to enhance the shoot elongation, root elongation and also the plant growth (Brown, 1974).

5.4.2 Number of nodules and chlorophyll content

Number of nodules and chlorophyll content was maximum in the treatment 12 followed by T_6 at 45 and 60 DAS and was

significantly superior over the control, whereas, the single inoculations of beneficial organisms showed increased nodule number and chlorophyll content over control. Similar increase in nodule number and chlorophyll content in several legumes have been reported by (Savalagi *et al.*, 1994; Balamurugan and Gunasekaran, 1996, Upadhayay *et al.*, 1999; Sharma *et al.*, 1999). The maximum nodule number due to inoculation of two or more beneficial organisms over single inoculation and uninoculated control has been reported (Iruthayathas *et al.*, 1983, Rao and Dhir, 1993; Alagawadi and Gaur, 1988; Balachandar and Nagarajan, 1999; Singh, 1996 and Sivaprasad and Rai, 1991).

The increase in the nodule number and chlorophyll content attributed can be ascribed to presence of rhizobia in the legume rhizosphere influencing the legume roots to secrete growth promoting sunstances, which in turn might have enhanced the growth of other three organisms *in situ* and a synergistic effect may have achieved in case of teatment 12 and 16. Enhancement of rhizobial growth, in case of treatment 12 (triple inoculation) might be due to inoculation of *Azospirillum*, where more root hairs become susceptible for rhizobial infection and also might be due to better provision for P-availability by phosphate solubilizers (Poi *et al.*, 1989). Moreover *Azospirillum* cells produce an excretable compound (1) which creates new infection sites (Plazinski and Rolfe, 1985).

The higher nodule number in JS-335, might be due to its vigorous growth habit resulting in deeper and wider penetration of roots and hence facilitating higher rhizobial infection.

5.4.3 Dry matter production

In accordance with the root and shoot growth the dry matter content in root and shoot as well as total dry matter of soybean plants was also enhanced due to inoculation of beneficial organisms. The maximum shoot and root dry weight was recorded in the treatment 12 consortia consisting of *Rhizobium*, *Azospirillum* and phosphate solubilizer however, which was on par with treatment 16 consirtia consisting of *Rhizobium*, *Azospirillum*, Phosphate solubilizers and general rhizobacteria increased root and shoot dry matter over uninoculated control, increasing combined inoculation further enhanced the root and shoot biomass. The increase in the plant dry weight due to single 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 was reported due to combined inoculation of *Rhizobium*, phosphate solubilizers, *Azotobacter, Azospirillum, Acetobacter* (Patil *et al.*, 1992; El-Mokadem *et al.*, 1989; Alagawadi and Gaur, 1988b; Prathibha *et al.*, 1994, Veena, 1999 and Devananda, 2000).

The increased plant dry weight in case of combined inoculations due to availability of a more balanced nutrition for plants by way of synergistic interactions and also might be due to release of growth regulators and ammonia from *Rhizobium* (Subba Rao, 1995) and continuos supply of P nutrition by solubilization of insoluble phosphates by phosphobacteria (Sperber, 1957). The increase may also be due to greater solubilization of P by the rhizosphere microorganisms would lead to better symbiotic N_2-fixation by the legumes and the latter one was found to have maximum contribution in increasing dry matter production.

5.4.4 Nutrient uptake (N and P)

In case of nutrient uptake, inoculation treatment 16 recorded significantly highest N and P uptake when compared with all other treatments. However, the N uptake and P uptake was lowest in uninoculated control, which increased with single inoculation, dual inoculation, triple inoculation and was maximum in treatment involving four organisms. The increase in N and P uptake due to single inoculation of N_2-fixers and phosphate solubilizers are reported (Sharma *et al.*, 1999; Parashar *et al.*, 1999; Mehta and Ram Mohan Rao, 1996; Defreitas *et al.*, 1997). The increase in N and P uptake due to combined inoculation of two or more organisms has been documented by several workers (Balamurgan and

Gunasekharan, 1996; Patil *et al.*, 1992; Devananda, 2000). These reports provided support to the results of present investigation.

The P-solubilizer used, capable of solubilizing a good amount of insoluble phosphate enhances the N uptake from soil solution and utilization of added N fertilizers improves symbiotic N_2-fixation of legumes and this may be attributed to the improvement in P-uptake from the soil solution, a process that enhances the nodulation and biological N_2 fixation (Subba Rao *et al.*, 1986). Maximum N uptake in the consortia of organisms might be due to improvement in effectiveness of legume *Rhizobium* symbiosis in presence of *Azospirillum* and P solubilizing microorganisms (Apte and Iswaran, 1971and Barea *et al.*, 1976.

5.4.4 Yield parameters

The yield parameters such as number of pods per plant and grain yield was recorded highest in treatment 12 followed by treatment 16. The increase in yield due to single inoculations with PGPR over control have been well reported (Defreitas *et al.*, 1997; Raut and Masood Ali, 1983; Prabhakaran *et al.*, 1999; Mehta and Ram Mohan Rao, 1996). However, the maximum increase in yield parameters due to dual and combined inoculation of PGPRs was documented (Patil

et al., 1992; Belimov *et al.*, 1995; Alagawadi and Gaur, 1988 and Prathibha *et al.*, 1994).

The increase in the yield parameters because the several factors such as release of growth promoting substances like IAA and GA, proliferation of beneficial organisms in the rhizosphere, control of plant pathogens in addition to phosphate solubilization and nitrogen fixation. The response might be due to probiotics of mixed culture of two or more types of organisms (Kundu and Gaur, 1984).

In treatment T_{16} where in combined inoculation of four organisms has give lesser yield compared to treatment T_{12} where in only three organisms were combindly inoculated. This may be because of competition for nutrients, which is limited under pot culture experiment. However, the same may not be extrapolated to field conditions.

Few reasons for highest yield in treatment receiving combined inoculation of three organisms are coated below.

1. The organism compatible to each other will only be selected hence there will be no competition among effective organisms.

2. The beneficial traits of each organism will be an additive factor resulting an overall complimentary or probiotic effect.

3. The mixture of nodulating, associative and free living organisms do not compete for the site of infection on plant roots.

4. The bio-control effects of cultures improve soil quality.

Summary

VI. SUMMARY

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

Plant growth promoting rhizobacteria (PGPR) such as *Rhizobium* (30 strains), *Azospirillum* (15 strains), phosphate solubilizers (18 strains) and general rhizobacteria (8 strains) isolated from different regions and they are characterized based on morphological, biological and physiological characteristics. Then they are screened for their beneficial traits such as N2-fixation, phosphate solubilization, production of plant growth promoting substances such as IAA, GA and biocontrol test. *Rhizobium* isolates are subjected for nodulation test as conformity test. Those nodulating isolates are selected for the further studies.

The amount of nitrogen fixed by the nitrogen fixing organisms indicate that *Rhizobium* were found to fix higher amount of nitrogen than *Azospirillum* in soybean. Phosphate solubilizers are subjected for phosphate solubilization test to know how much amount of tricalcium phosphate solubilized in the broth indicating efficiency of each isolates. The amount of P released from the TCP by the phosphate solubilizing bacteria was ranged from 19 to 47.5 mg/100 ml broth.

Similarly, the *Azospirillum*, P-solubilizers were tested for production of plant growth promoting substances like, IAA and GA. All the isolates of phosphate solubilizers produced both IAA and GA. All the isolates of *Azospirillum* produced IAA whereas, three isolates were incapable of producing GA. In case of general rhizobacteria except two strains all produced IAA and GA.

For bio-control test only efficient isolates were taken from each group and tested against the pathogen (*Sclerotium*). Out of five isolates selected from each group, 2 isolates from *Rhizobium* and *Azospirillum* and one isolate from phosphate solubilizer were capable of suppressing the pathogen. Whereas, All the efficient three isolates selected from general rhizobacteria, showed negative effect in bio-control test.

By considering all these beneficial traits one efficient strain was selected from each group to know the combined effect of these growth promoting rhizobacteria on the growth and yield of soybean.

All the inoculation treatments showed better plant growth, yield and nutrient uptake compared to uninoculated control. Among the inoculation treatments, single inoculations performed better than uninoculated control whereas, dual inoculations performed better over single inoculation treatments. Combined inoculation of three beneficial organisms

(*Rhizobium* + *Azospirillum* + PSB) was superior over dual, single inoculants and control. However, this was on par with the combined inoculation of four beneficial organisms (*Rhizobium* + *Azospirillum* + phosphate solubilizers + general rhizobacteria) with respect to plant growth, yield and nutrient uptake. The results of our studies indicate that the combined inoculations involving three or more beneficial organisms exerts more favourable effect on growth and productivity of soybean than dual or single inoculations. Results can be extrapolated to field conditions. The results of combination of four organisms were on par with combination of three organisms and hence the desired combination of *Rhizobium* + *Azospirillum* + PSB was producing maximum favourable influence on growth and yield of soybean and could be recommended for field applications in soybean.

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Appendix

APPENDIX- I

COMPOSITION OF MEDIA

Composition of ketolactose medium (Bernaertz and Deley, 1963).

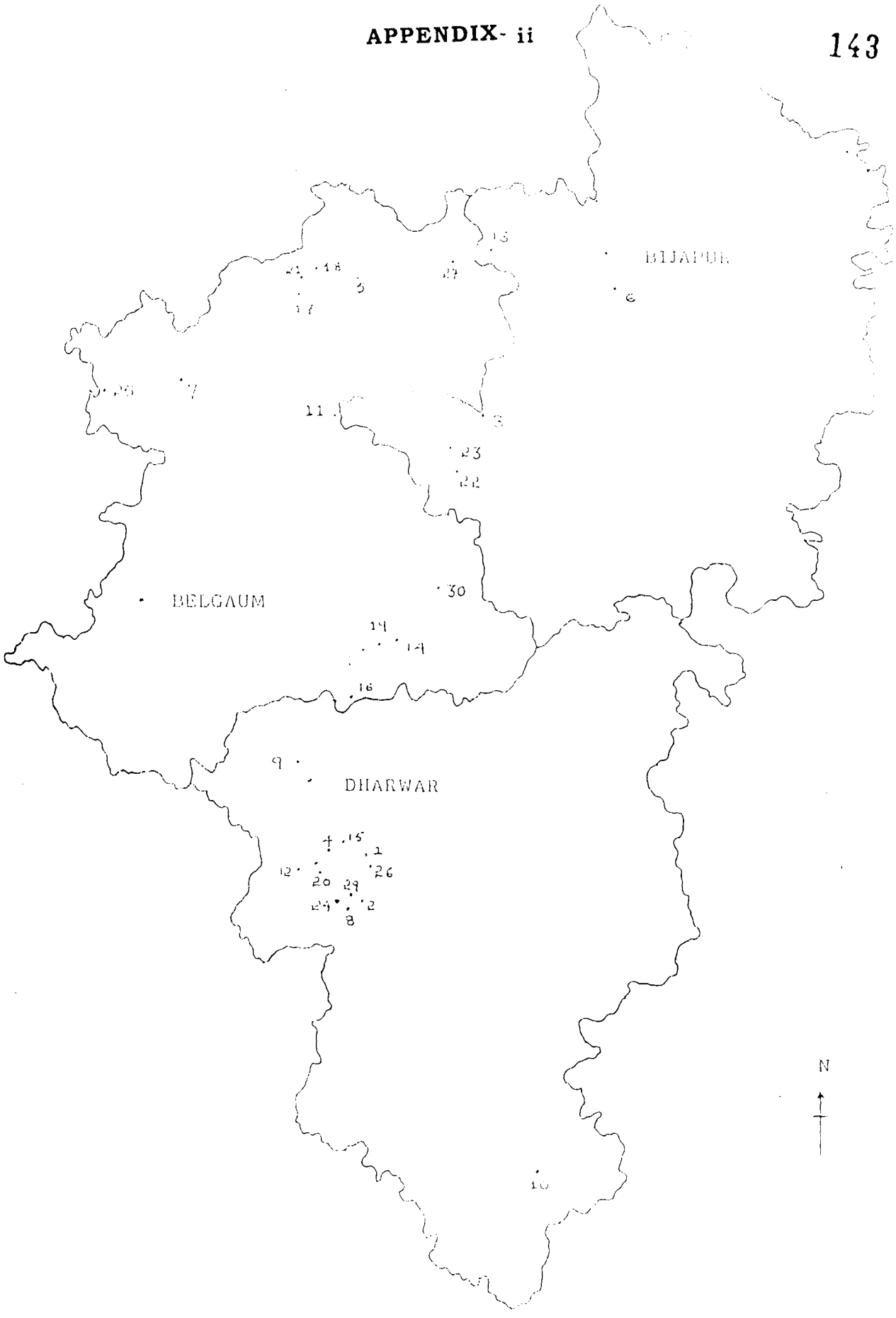
Lactose	- 10.0 g	NaCl	- 0.2 g
K_2HPO_4	- 0.5 g	$FeCl_3$	- 0.01 g
$CaCl_2$	- 0.2 g	Distilled water	- 1000 ml
Yeast extract	- 0.5 g	Agar	- 15.0 g
$MgSO_4 . 7H_2O$	- 0.1 g	pH	- 6.8

Composition of Hofer's broth (Hofer, 1935)

K_2HPO_4	- 0.50 g
$MgSO_4 . 7H_2O$	- 0.20 g
NaCl	- 0.10 g
$CaCO_3$	- 0.05 g
Yeast extract	- 3.0 g
Mannitol	- 10.0 g
Distilled water	- 1000 ml
1 N NaoH	- 28 ml
pH	- 11

Composition of Plant nutrient solution (Jensen, 1942)

K_2HPO_4	- 0.2 g	H_3BO_3	- 0.05 g
$MgSO_4 . 7H_2O$	- 0.2 g	$MnSO_4$	- 0.05 g
NaCl	- 0.2 g	$ZnSO_4$	- 0.005 g
$FeCl_3$	- 0.1 g	pH	- 6.5-7.0
Water	- 1000 ml		



Map of areas from where isolates collected.

STUDIES ON GROWTH PROMOTING RHIZOBACTERIA ASSOCIATED WITH SOYBEAN [*Glycine max* (L.)]

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ABSTRACT

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

Plant growth promoting rhizobacteria (PGPR) such as *Rhizobium* (30 strains), *Azospirillum* (15 strains), phosphate solubilizers (18 strains) and general rhizobacteria (8 strains) isolated from different regions and they are characterized based on morphological, biochemical and physiological characteristics. Then they are screened for their beneficial traits such as N_2-fixation, phosphate solubilization, production of plant growth promoting substances such as IAA, GA and biocontrol test. By considering all these beneficial traits one efficient strain was selected from each group to know the combined effect of these growth promoting rhizobacteria on the growth and yield of soybean.

All the inoculation treatments showed better plant growth, yield and nutrient uptake compared to uninoculated control. Among the inoculation treatments, single inoculations performed better than uninoculated control whereas, dual inoculations performed better over single inoculation treatments. Combined inoculation of three beneficial organisms (*Rhizobium* + *Azospirillum* + PSB) was superior over dual, single inoculants and control. However, this was on par with the combined inoculation of four beneficial organisms (*Rhizobium* + *Azospirillum* + phosphate solubilizers + general rhizobacteria) with respect to plant growth, yield and nutrient uptake. The results of our studies indicate that the combined inoculations involving three or more beneficial organisms exerts more favourable effect on growth and productivity of soybean than dual or single inoculations. Results can be extrapolated to field conditions. The results of combination of four organisms were on par with combination of three organisms and hence the desired combination of *Rhizobium* + *Azospirillum* + PSB was producing maximum favourable influence on growth and yield of soybean and could be recommended for field applications in soybean.