RESPONSE OF DIFFERENT CROPS TO INOCULATION WITH PLANT GROWTH PROMOTING RHIZOBACTERIA

ANGAW TSIGIE

DIVISION OF MICROBIOLOGY
INDIAN AGRICULTURAL RESEARCH INSTITUTE
NEW DELHI-110 012

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Dedicated to
my loving Mother
Woizero Ertib Tadagi
and
my darling wife
Woizero Masho Wolde Tsadik

RESPONSE OF DIFFERENT CROPS TO INOCULATION WITH PLANT GROWTH PROMOTING RHIZOBACTERIA

A thesis

by

ANGAW TSIGIE

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Approved by :

Chairman :	Dr. K.V.B.R. Tilak	
Members :	Dr. A.K. Saxena	
	Dr. G. Narayanasamy	18/0[illegible]07
	Dr. D.V. Singh	
	Dr. J.M.S. Mathur	

Dr. K.V.B.R. Tilak
FNASS, FNASc
Head

DIVISION OF MICROBIOLOGY,
INDIAN AGRICULTURAL RESEARCH INSTITUTE,
NEW DELHI - 110 012

CERTIFICATE

This is to certify that the thesis entitled **"Response of Different Crops to Inoculation with Plant Growth Promoting Rhizobacteria"**, submitted to the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** is a record of *bona fide* research work carried out by **Shri Angaw Tsigie** under my personal supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that assistance and help received, and source of information availed during the course of this investigation have been duly acknowledged.

Place : New Delhi
Date : July 28 , 2001

(K.V.B.R. TILAK)
Chairman
Advisory committee

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1.0 INTRODUCTION

Microorganisms are both foes and friends of man. Though man understands microbes as evils due to their devastating effects on his crop plants, animals and as well as on himself, he had been taking advantage of their enormous beneficial contribution in this world. Some of the microbes that inhabits soils, rivers, lakes oceans and air are beneficial to man.

Microorganisms play a very important role in the field of agriculture. They are useful in maintaining soil texture and fertility. Essential elements and plant nutrients are recycled by the help of soil microorganisms. Even though fertilizers applied by man have become major agricultural input, the role of microorganisms in nitrogen fixation and biochemical cycling of organic matter cannot be under estimated. Their important contribution to the livelyhood of man aroused tremendous interest in studying the activities and their potential utilization for the benefit of man.

Microbiologists such as Helriegel and Wilforth in 1888 showed conclusively that legumes could utilize atmospheric nitrogen and that their utilization was dependent upon bacteria present in the nodules. Subsequent studies led to the discoveries of *Rhizobium, Clostridium, Azotobacter, Azospirillum* and blue green algae as very important N_2 - fixing soil organisms.

Later on, in 1904, Hiltner made some interesting observations regarding the relationship between soil microorganisms and plant roots. He found that certain microorganisms showed increased activity close to the roots of cultivated plants. The portion of soil adjacent to the root influenced by it is called "*rhizosphere*". Hiltner's observation opened a door to microbiologists to study the relationship of plant species and soil microorganisms in the rhizosphere. The term **plant growth promoting rhizobacteria** (PGPR) has been introduced (Kloepper *et al.*, 1980) for the bacteria that colonize the plant roots and stimulate plant growth and crop yield. PGPR are found to increase plant yield by 10 to 30% in non-legumes. Many bacteria belonging to genera *Pseudomonas, Bacillus, Azospirillum, Azotobacter, Enterobacter, Serratia* and *Arthrobacter* have a vast promise as PGPR and are now used in agriculture as bioinoculants. A subset of PGPR strains which enhance the nodulation of legumes by rhizobia have been designated as **nodulation promoting rhizobacteria** (NPR).

Successful crop production is highly dependent on the availability of various nutrients. Among these, nitrogen (N) is the most needed one, which is limiting in Indian soils. Emphasizing the gravity of the problem Mehla and Gupta (1998) wrote that almost all the Indian soils are deficient in N and no crop can be raised without its application. Nitrogenous fertilizers constitute the major components among the chemical fertilizers used in agriculture. It is estimated that the crops removing about 9-10 million tonnes more N + P_2O_5 + K_2O every year than the total additions through fertilizers in India (Tandon, 1995). Moreover, during the diagnostic survey of rice-wheat area of Kurushetra and Karnal districts of Haryana, it was observed that many farmers were applying more N fertilizers than the recommended dose to harvest good yield of both rice and wheat crops (Harrington *et al.*, 1993). Mehla and Gupta (1998) had come out with the result that the fertilizer N dose for 'WH 542' and 'WH 2329' wheat varieties under rice-wheat cropping system need to be increased to about 200 kg N/ha to get their potential yields and to meet the future challenges of food demand.

Phosphorus (P) is one of the important and sometimes it may be the first limiting nutrient for plant growth. P nutrition benefits the plant by producing deeper and abundant roots. Hence, the supply of this element to plant is essential for achieving optimum crop yield. It is supplied through phosphatic fertilizers, animal manures, plant residues, domestic organic wastes and rock phosphate. The introduction of the most efficient P - solubilizing microorganisms (PSM) in the rhizosphere of crop and soil increases the availability of phosphorus from insoluble sources of phosphate and also increases the efficiency of phosphate fertilizers such as superphosphate and rockphosphate. The phosphate uptake was enhanced and the yields were increased by 10 to 50% with different crops. A combination of rock phosphate and PSM was found to be more effective than using rock phosphate alone particularly in soils having neutral to alkaline pH. In general, 40% of superphosphate could be saved by rockphosphate and PSM (Jauhri, 1998).

It is very clear that NPK are not only quite important but also limiting crop production and consequently affecting our livelihood. It is also obvious, that the modern agriculture is heavily dependent on chemical fertilizers to meet the food demands of ever increasing population. The production of chemical fertilizer is based on non-renewable and constantly depleting petroleum based feed stocks. Progressive depletion of major plant nutrients in soil due to intensive cultivation has necessitated the use of higher dose of chemical fertilizers particularly in tropical soils where the organic matter content is very low. This huge drain on nutrients will continue to impoverish the soils unless these are replenished by natural or artificial means as concluded by Gupta *et al.* (1998).

Application of high doses of chemical fertilizers may temporarily help to increase crop production and may relieve the problem of demand and supply and ever increasing population. However, this may turn bitter and with highly regrettable consequences where soil fertility will be depleted or become acidic and devoid of macro and micronnutrients for crops to grow and microorganisms to proliferate. Thus it is absolutely necessary to awake timely and be able to use ecofriendly inputs such as beneficial PGPR and save our 'currency', the soil, and its consituents.

Three new bacteria namely *Proteus vulgaris, Klebsiella planticola* and *Bacillus subtilis* isolated from the rhizotic zones and above ground of sunflower have been reported to increase yield of sunflower (Malik *et al.,* 1995). These cultures have not been characterized biochemically and their effect on wide spectrum of crops such as cereals, legumes and oil seeds have not been studied. Therefore, the present investigation is concerned with the following objectives :

1. Cultural and biochemical characterization of the three plant growth promoting rhizobacteria namely *Proteus vulgaris*, *Klebsiella planticola* and *Bacillus subtilis.*

2. To study the influence of PGPR strains on growth and yield of
    - (a) cereal crops
    - (b) oil seed crops and
    - (c) leguminous crops

3. To investigate the establishment of PGPR strains in the rhizosphere of few selected crops.

4. To develop carrier based inoculant and to study the survival of PGPR strains.

2.0 REVIEW OF LITERATURE

2.1 Rhizosphere and Soil microorganisms

Soil is the natural growth media for living plants and microorganisms. The physical and chemical properties of soil have a dominant role to play on the soil microorganisms. Bacteria are the most dominant group of microorganisms in the soil and probably equal to one half of the microbial biomass present there in. The most abundant bacterial forms or genera present in the soil are *Pseudomonas, Arthrobacter, Clostridium, Bacillus, Micrococcus, Flavobacterium, Chromobacter, Sarcina, Enterobacter, Corynebacterium, Mycobacter* etc. (Mishra 1996; Rangaswami and Bagyaraj, 1998; Subba Rao, 2000). Soil is also an environment in which the underground parts of the plant have their abode. As they grow through the soil, the condition of the soil in the immediate vicinity of the root witness a drastic change in various ways. The microhabitat of the soil in close proximity of root changes and this has an impact on the microorganisms residing in the region. This area of enhanced activity surrounding living roots that is composed of soil particles and active communities of soil microorganisms is called the *rhizosphere* (Hiltner, 1904). This environment is richer in nutrients, and its microbial communities differ from those present in soil not influenced by the roots (Alexander, 1977). It is now recognized that the '*rhizosphere effect*' is due to the root exudates which attract some soil microorganisms. The width of the zone of soil thus influenced by the root varies with the plant species its age and cultural conditions, soil conditions, environmental conditions etc. (Rangaswami and Bagyaraj, 1998).

Some bacterial species living in the rhizosphere can affect plant growth in either a positive or in a negative way. Rhizosphere bacteria that favourably affect plant growth and yield of commercially important crops are denominated as **Plant Growth Promoting Rhizobacteria** (PGPR) (Kloepper and Schroth, 1978). Beneficial effects of rhizospheric bacteria have most often been based on increased plant growth, faster seed germination, better seedling emergences, enhanced nodulation and nitrogen fixation in leguminous crops and suppression of diseases. As a consequence, PGPR have been further divided into subsets like emergence promoting rhizobacteria (EPR); nodulation promoting rhizobacteria (NPR) and disease suppressing rhizobacteria (DSR) (Kloepper *et al.,* 1986b). However, there can be considerable overlap among these subsets. For instance, PGPR that act through biological control can also enhance germination or growth. In recent years, many reviews have appeared dealing with PGPR isolation, screening, ecology, physiology and their use as agrobiotechnological inoculants (Glick 1995; Lazarovits and Nowak, 1997; Saxena *et al.,* 2000).

2.2 Isolation and identification of PGPR

In the last two decades several attempts have been made by different workers to isolate bacteria from the rhizotic (rhizosphere, rhizoplane and endorhizosphere) zones of different crop plants (Ladha *et al.,* 1983; Glick *et al.,* 1995; Chabot *et al.,* 1996; de Frietas and Germida 1997; Han and New, 1998).

Acid and gas producing nitrogen fixing bacteria were isolated from rice roots and leaf sheaths by using semisolid glucose yeast extract (GYE) medium. Following biochemical tests and cross reactions in gel immunodifusion, two different serogroups; *Enterobacter cloacea* and *Klebsiella planticola* were identified (Ladha *et al.,* 1983).

Employing the semisolid nitrogen free media, a total of 285 strains of *Azospirillum* were isolated from soils from seven geographic regions in New South Wales, Australia. By combining amplification and restriction analysis of 16S rDNA (ARDRA) patterns with serological, morphological and biochemical results, all isolates were found to be *A.brasilense* and *A. lipoferum* (Han and New, 1998).

Cattelan *et al.* (1998) isolated 1131 bacteria from bulk soil and the rhizosphere of soybean. On the basis of fatty acid methyl ester (FAME) identification, 60 isolates representing 15 different species from eight different genera were selected. The most common genera were *Pseudomonas, Burkholderia, Bacillus* and *Alcaligenes* (Cattelan *et al.,* 1998).

Using the same technique of FAME identification, phosphate solubilizing isolates belonging to *Bacillus brevis, B. megaterium, B. polymyxa, B. sphaericus, B. thuringiensis* and *Xanthomonas maltaphilia* were isolated and identified (de Frietas and Germida, 1997).

A rapid and novel procedure for the isolation of PGPR was described by Glick *et al.* (1995). This method entails screening soil bacteria for the ability to utilize the compound 1 - aminocyclopropane - 1 - carboxylate (ACC) as a sole N source, a trait that is a consequence of the presence of the activity of the enzyme ACC deaminase. This trait appears to be limited to soil bacteria that are capable of stimulating plant growth. This method appears to be rapid and should replace the otherwise slow and tedious process of testing individual strains for their ability to promote plant growth (Glick *et al.,* 1995).

Isolation were made from the soybean rhizosphere using the dilution plate count method. *Enterobacter* and nonspecific bacterial populations were a major component of the rhizosphere microflora. Gram-negative bacteria and actinomycetes were next in abundance, followed by pseudomonads (Hicks and Laynachan, 1988).

Bacteria were isolated from the rhizosphere of 9-month old winter wheat plants (cv. Norstar) using following media : (i) combined carbon (CC) for isolation of putative nitrogen fixers (ii) Mac Conkey's (MAC) and MAC plus 100 $\mu g\ ml^{-1}$ carbenicillin (MACC) to separate presumptive N_2-fixing Enterobacteriaceae and non Enterobacteriaceae (Bagley and Seidler, 1978; Rennie 1981); and (iii) tryplicase soy agar (TSA) for total soil bacteria counts. A total of 300 isolates were obtained which were further screened for their ability to grow on King's B medium (KB) and KB supplemented with antibiotics (chloramphenicol, cycloheximide, novobiocus and penicillin) to make it selective for fluorescent pseudomonads (de Freitas and Germida, 1990).

Forty five gram negative bacterial strains were isolated from the rhizosphere of sunflower and screened for their growth promoting activity (Fages and Arsac, 1991). Three growth promoting rhizobacteria namely *Klebsiella planticola, Proteus vulgaris* and *Bacillus subtilis* were isolated from root region and root surfaces of sunflower (Malik *et al.,* 1995).

Rhizobacteria from rhizosphere of chickpea plants were isolated using fungal cell wall material as carbon source for growth. A total of 36 isolates were obtained, of which 5 were found to inhibit the growth of *Fusarium oxysporum* and *Rhizoctonia bataticola* (Khot *et al.,* 1996).

A number of diazotrophic bacteria were isolated from various root fractions of kallar grass, a highly salt-tolerant plant species. These belong to the genera of *Azospirillum, Azoarcus, Enterobacter, Klebsiella* and *Zoogloea* (Bilal and Malik, 1987; Bilal *et al.,* 1990; Zafar *et al.,* 1987).

Attempt was made to isolate rhizobacteria from the rhizotic zones of greengram using seven selective and four non-selective media. A total of 121 bacteria were isolated of which Gram-ve bacteria accounted for 65%. The dominant genera were *Pseudomonas, Bacillus, Enterobacter, Proteus* and *Klebsiella* (Gupta *et al.,* 1998a).

Nutrient agar supplemented with glucose was used to isolate bacteria from rhizosphere of wheat. Characterization of these isolates revealed the presence of *Kurthia* sp. a Gram+ve, urea hydrolysing bacteria (Malik *et al.,* 1999).

2.3 Mechanisms of plant growth promotion

The mechanisms by which PGPR promote plant growth are not fully understood but are thought to include (i) the ability to produce or change the concentration of the plant hormones-indole acetic acid (IAA ; Mordukhova *et al.,* 1991), gibberellic acid (Mahmoud *et al.,* 1984), cytokinins (Tien *et al.,* 1979), and ethylene (Arshad and Frankenberger, 1991; Glick *et al.,* 1995); (ii) asymbiotic N_2 fixation (Boddey and Dobereiner, 1995; Kennedy *et al.,* 1997); (iii) antagonism against phytopathogenic microorganisms (e.g *Fusarium* spp; Scher and Baker, 1982) by production of siderophores (Scher and Baker, 1982), β - 1, 3 - glucanase (Fridlender *et al.,* 1993), chitinases (Renwick *et al.,* 1991), antibiotics (Shenahan *et al.,* 1992), and cyanide (Flaishman *et al.,* 1996); and (iv) solubilization of mineral phosphates and other nutrients (Sperber 1958a, 1958b; De Freitas *et al.,* 1997).

2.4 Plant growth regulating substances

Many rhizosphere bacteria produce IAA (Indole acetic acid) in culture media specially in the presence of tryptophan (Lynch, 1976; Ek *et al.*, 1983; Strzelczyk and Pokojska-Burdziej, 1984), in the rhizosphere and rhizoplane of forage grasses and many economically important cereals like wheat, barley and pearl-millet (Brown 1972; Tien *et al.*, 1979), vegetables, tomato and bean plants (Jackson, 1964; Barea and Brown, 1974) under cultural conditions.

Tien *et al.* (1979) found production of plant growth promoting substances in liquid culture by the N fixing *Azospirillum brasilense*. The bacteria produced small amounts of IAA, gibberellin and cytokinin-like substances.

Barea *et al.* (1976) examined culture supernatant of 50 phosphate - dissolving bacteria, isolated from rhizospheres of crop plants for IAA, gibberellins and cytokinins. Twenty bacteria were found to synthesize all three types of plant hormones; 43 produced IAA, 29 produced gibberellins and 45 cultures produced cytokinin-like substances.

Prikryl *et al.* (1985) reported production of IAA and some other auxins in liquid culture of *Pseudomonas cepacia* and *P. fluorescens* isolated from maize and bean rhizosphere.

Loper and Schroth (1986) have reported accumulation of IAA in the culture filtrate of rhizobacterial isolates from the rhizosphere of sugar beet. Bacterization of seeds with two isolates 7SR5 and 7SR13 producing highest concentration of IAA (5-10 $\mu g\ ml^{-1}$) resulted in reduced root elongation and increased shoot : root ratio of sugar beet. Similar effects were observed by seed bacterization with *Pseudomonas syringae* pv. *savastonoi,* strains 2009 and 2009-6, which produced IAA. In contrast, derivative strains 2009-3 and 2009-561 (derived from strains 2009 and 2009-6, respectively), deficient in IAA production, did not show such effects.

Brown and Burlingham (1968) detected the presence of three gibberellin-like substances in the culture of *Azotobacter chroococcum* strain A6. When an inoculum of *Azotobacter* was added to seeds or roots, the later development of tomato plants was altered, possibly because it was taken up by the seedlings at a critical stage of vegetative and reproductive primordia differentiation.

Arshad and Frankenberger (1988) while studying the production of ethylene (C_2H_4) by the soil fungi *Acremonium falciforme* showed that microbially produced C_2H_4 can affect plant growth of etiolated pea seedlings. Etiolated pea seedlings presented a classical triple response, which includes reduction in elongation, swelling of the hypocotyl and a change in the direction of growth (horizontal), when *A. falciforme* was used as an inoculum. Further studies by Frankenberger and Arshad (1990) showed production of plant growth regulators (PGRs) by many soil microorganisms in the presence of suitable precursors. In laboratory studies, the microbial biosynthesis of auxins, cytokinins and ethylene in soil was monitored.

Dubeikovsky *et al.* (1993) demonstrated the existence of positive effect of bacterial IAA on plants after inoculation of black current soft wood cuttings by a recombinant *Pseudomonas* strain. Production of bacterial indole - 3 - acetic-acid (IAA) was shown to have a stimulatory effect on the development of black current soft wood cuttings by a recombinant strain.

Twenty two *Bacillus* spp. isolates from the rhizosphere of *Phaseolus vulgaris* 'contender' produced significant amounts of IAA when grown in a liquid culture medium supplemented with 100 µg L - tryptophan L^{-1}; less IAA was produced in absence of L-tryptophan. The amount of IAA produced by various strains varied from 0.40 to 4.88 µg mL^{-1}.

In liquid culture of *Azospirillum brasilense* Cd, the concentration of IAA increased rapidly with the beginning of stationary phase. This suggested that the high increase in IAA production in stationary phase is the expression of an overall change in cell metabolism when the carbon source is exhausted (Omay *et al.,* 1993).

Rhizobium leguminosarum strains shown to improve growth of canola and lettuce can serve as PGPR. Their growth promotive effects involve the production of IAA and cytokinin (Noel *et al.,* 1996).

Malik *et al.* (1997) observed IAA production in five strains of PGPR. These PGPR when inoculated in Kallar grass and rice resulted in increased acetylene reducing activity (ARA). But no correlation was found between IAA production and ARA.

During the last few years, a new mechanism of plant growth promotion involving ethylene has been proposed. It was demonstrated that a small number of soil bacteria contains the enzyme ACC deaminase (Klee *et al.,* 1991). This enzyme hydrolyzes ACC, the immediate precursor of ethylene in plants, to yield ammonium and α - ketobutyrate. Bacteria possessing ACC deaminase activity may therefore grow using ammonium as the sole nitrogen source. The beneficial rhizobacteria *Pseudomonas putida* strain GR 12-2 stimulates root elongation of different plants. Later, it was found that this bacteria contains ACC deaminase (Jacobson *et al.*, 1994). Mutants of this strain lacking ACC deaminase activity were not able to promote root elongation of canola seedlings, implicating this enzyme in the mechanism of root growth promotion by this bacteria (Glick *et al.*, 1994a, 1994b). A model of the possible role of ACC deaminase in root elongation is shown by Glick *et al.* (1994a) ; following the binding of the bacteria to the seed coat, ACC is hydrolyzed by ACC deaminase. Thus lowering the level of ethylene during imbibition and then promoting root elongation.

A review on the formation of hydrocarbons by microorganisms indicated that while ethylene is a common product of fungi, the soft rot bacterium, *Pseudomonas solanacearum* has also been shown to produce the gas in pure culture. More recently its formation by other bacteria has been demonstrated.

Experimental results on the effect of wild-type and mutant PGPR (*P. putida*) in the rooting of mungbean cuttings showed that treated cuttings had a significant higher number of roots compared with cuttings rooted in water. It was also clear that ethylene is involved in the initiation and elongation of adventitious roots in mungbean cuttings (Mayak *et al.*, 1999).

Enhancement of symbiotic nitrogen fixation and plant growth have been reported due to inoculation of Vitamin B 12 secreting *Pseudomonas* strain 267 in growth medium (Derylo and Skorupska, 1993).

2.5 Asymbiotic nitrogen fixation

Root-associated bacteria capable of fixing nitrogen occur regularly in diverse soils which vary widely in nitrogen content and the capability to metabolize N_2 provide a strong competitive advantage in soils of low organic nitrogen content.

Non-symbiotic nitrogen-fixing bacteria are important in the natural environment. Long-term nitrogen-balance studies have shown them to be capable of significant rates of nitrogen accumulation (Day *et al.,* 1975; Neyra and Dobereiner, 1977). Several studies indicate N_2 fixation rates of about 30-40 kg ha^{-1} $year^{-1}$ by associative bacteria.

Sprent (1979) presented an extensive list of species currently known to fix nitrogen. Common genera capable of fixing nitrogen include *Azospirillum, Azotobacter, Bacillus, Clostridium, Derxia* and *Klebsiella.* These are commonly designated "free-living" bacteria, since they are able to exist in the soil and reduce nitrogen without entering into a symbiotic association with plants.

There have been many research efforts to demonstrate significant biological N_2 fixation by free living diazotrophs in the rhizosphere and on the surface of roots (Boddey and Dobereiner, 1994; Okon and Labandera-Gonzalez, 1994a; Saxena and Tilak, 1998).

Nitrogen fixation activities measured in agronomically important plants (wheat, maize rice, sorghum) inoculated and colonized by *Azospirillum* and *Azotobacter* under greenhouse conditions or in field have generally been low (Okon and Labandera-Gonzalez, 1994a). Using extensive estimations and measurements of N_2-fixing activities in these rhizosphere associations, by using a variety of methodologies such as the acetylene reduction assay and the ^{15}N dilution technique (*in situ* and in the field), it was estimated that associative symbioses could contribute to about 5 kg

N ha^{-1} in *Azospirillum* inoculated wheat, sorghum and maize fields (Boddey and Dobereiner, 1994; Okon and Labandera-Gonzalez, 1994a).

It has been reported that in some cultivars of sugarcane of Brazil and of kallar grass in waterlogged salty soils in Pakistan, substantial quantities of nitrogen (about 50 kg N ha^{-1}) can be fixed by free living diazotrophs associated with the plant *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* are N_2-fixing bacterial endophytes that might be responsible for the proposed high N_2-fixation in sugarcane (Boddey and Dobereiner, 1994). Species of the genus *Azoarcus* are endophytes frequently isolated from roots of kallar grass (Reinhold-Hurek and Hurek, 1993).

2.6 Antagonism against phytopathogenic fungi

During the last two decades, several examples of rhizobacteria capable of providing substantial disease control in the field have been reported (Saxena *et al.,* 2000). Many bacterial genera have shown their potential for biocontrol both under *in vitro* and *in vivo* conditions. *Agrobacterium* (Kerr, 1972, 1980; Thomson, 1987). *Arthrobacter* (Mitchell and Hurwitz, 1965), *Alcaligenes* (Martinetti and Loper, 1992), *Azotobacter* (Meshram and Jagar, 1983), *Bacillus* (Asaka and Shoda, 1996; Fiddaman and Rossall, 1995; Hwang, 1994; Michereff *et al.,* 1994a; Sharga, 1997; Silo-Suh *et al.,* 1994), *Escherichia coli* (Roberts *et al.,* 1997a, b), *Enterobacter* (Costa and Loper, 1994; Roberts *et al.,* 1997b), *Pseudomonas* (Dowling and O'Gara, 1994; Gomes *et al.,* 1996; Hallman *et al.,* 1996; Hebber *et al.,* 1992; Kloepper *et al.,* 1980; Moulin *et al.,* 1994, 1996; Pierson and Weller, 1994; Thara and Gnanamanickam, 1994), *Burkholderia* (King and Parke, 1993; Roberts *et al.,* 1997a,b). *Rhizobium* and *Bradyrhizobium* (Chakraborty and Purkayastha, 1984; Tu, 1980), *Serratia* (Berg and Behl,

1997) and *Stenotrophomonas* (Berg and Behl, 1997) were found to be potent for suppression of soil-borne fungal pathogens. Many of these biocontrol agents exhibited their effectiveness under field conditions also.

Agrobacterium radiobacter strain 84 was the first bacterium used commercially for biocontrol of crown gall caused by *A. tumefaciens* and got worldwide success (Kerr, 1980).

Martnetti and Loper (1992) reported that *Alcaligenes* sp strain MFAI inhibited microconidial germination and germ tube elongation of *Fusarium oxysporium* f. sp. *dianthi* and also reduced the severity of disease presumably as a result of siderophore production.

The usefulness of *Bacillus* as a source of antagonist for many plant pathogens (Campbell, 1989) is well known. Several potent strains from different species of *Bacillus* have been tested on a wide variety of plant species for their ability to control several diseases. *Bacillus* has ecological advantages because it produces endospores that are tolerant to extreme environmental conditions such as heat and desiccation. The greatest interest is *Bacillus subtilis* A 13, which was isolated from lysed mycelium of *Sclerotium ralfsii* by Broadbent *et al.* (1971). It was found to be antagonistic to several pathogens and as seed inoculant, increased the yield of carrots by 48%, oats by 33% (Merriman *et al.*, 1974) and peanuts by 37% (Turner, 1987). Since, 1983 it has been sold as a bioinoculant under the trade name QUANTUM - 4000 (Turner, 1987). *Bacillus* spp. and actinomycetes share several features that make them attractive for biological control agents including their abundance in soil and the production of various biologically potential metabolites active against a range of fungi (Silo-Suh *et al.*, 1994). In this respect *Bacillus cereus* UM 85 has shown a wide range of biological effects on plants. It was found to protect alfalfa seedlings from damping-off caused by *Phytophthora medicaginis* (Handelsman *et al.,* 1990), tobacco seedlings from *Phytophthora nicotianae* (Handelsman *et al.,* 1991), cucumber fruits from rot caused by *Pythium aphanidermatum* (Smith *et al.,* 1993), and peanut from *Sclerotinia minor* (Phipps, 1992). At least supprssion of alfalfa damping-off was due to the production of two fungistatic antibiotics (Silo-Suh *et al.,* 1994). Likewise, *Bacillus subtiis* RB14, produces antibiotics iturin and surfactin and could suppress *Rhizoctonia solani* damping-off of tomato (Asaka and Shoda, 1996). *Bacillus* BS 153 was able to prevent chocolate spot symptoms (causal agents *Botrytis cinerea* and *B. fabae*) on faba beans both in green house and field studies (Sharga, 1997). *Bacillus subtilis* strain could reduce root rot of lentil caused by *Fusarium avenaceum* (Fr.) Sacc (Hwang, 1994), northern leaf blight of corn (causal organism *Exserohilum turcicum*) (Reis *et al.,* 1994) rust infection of safflower caused by *Puccinia carthami* (Tosi and Zazzerini, 1994) *Fusarium* wilt of cotton caused by *Fusarium oxysporum* (Zhang Jin Xu *et al.,* 1995) Yam leaf spot disease (causal organism *Curvularia eragrostridis*) (Michereff *et al.,* 1994b) and anthracnose fruit rot of chilli caused by *Colletotrichum capsici* and *C. gloeosporioides* (Sariah-Meon, 1995). *Bacillus subtilis* and other *Bacillus* spp. were also reported to control post-harvest diseases of apple caused by *Penicillium expansum* and *Botrytis cinerea* (Sholberg *et al.,* 1995).

Enterobacter cloaceae, a potent root coloniser, was reported as biocontrol agent against *Pythium ultimum,* a prevalent phytopathogen that causes damping-off of many crop plants (Costa and Loper, 1994; Howell *et al.,* 1988; Nelson, 1988). Nelson (1988) reported the potentiality of *Enterobacter cloacae* strain ECct-501 to prevent pre-emergence damping-off of cotton. Howell *et al.* (1988) applied *E. cloacae* strain ECH-1, which produced ammonia, to control the damping-off of cotton caused by *P. ultimum.* Costa and Loper, (1994) evaluated and characterised that siderophore of *E. cloacae* strain EcCT-501 was responsible for disease suppression. *E. cloacae* strain 501 R3, a rifampicin resistant mutant of EcCT-501 was demonstrated to control damping-off of cucumber by *Pythium ultimum* both alone and in combination with *Burkholderia cepacia* (Roberts *et al.,* 1997b).

Fluorescent pseudomonads have revolutionized the field of biological control of soil-borne plant pathogenic fungi. During the last 25 years, they have emerged as the largest potentially most promising group of plant growth promoting rhizobactreia involved in the biocontrol of plant diseases (Barbosa *et al.,* 1995; Burr *et al.,* 1978; Fravel, 1988; Gomes *et al.,* 1996; Pierson and Weller, 1994; Wei *et al.,* 1996). Fluorescent pseudomonads have received the most attention for several compelling reasons. First, fluorescent pseudomonads readily colonize roots in nature, where they are frequently the most common of microorganisms (Weller, 1988). The simple nutritional requirement and the ability to use many carbon sources that exude from roots and to compete with indigenous microflora, may explain their ability to colonize the rhizosphere (Mazzola and Cook, 1991). Additionaly, pseudomonads are amenable to genetic manipulation. These characteristics make them useful vehicle for the delivery of antimicrobial and insecticidal compounds and plant hormones to the rhizosphere. O'Sullivan and O'Gara (1992) reviewed the traits of fluorescent pseudomonads such as production of antibiotics, hydrogen cyanide, siderophore which are involved in suppression of plant root pathogens.

There are numerous examples of biocontrol of several devastating fungal plant pathogens of important crops by fluorescent pseudomonads and has been reviewed from time to time (Cook, 1993; O'Sullivan and O'Gara, 1992; Weller, 1988). Fluorescent *Pseudomonas* spp. have been implicated in the control of several wilt diseaes caused by *Fusarium* spp. (Chen *et al.*, 1995; Hebbar *et al.*, 1992; Pal, 1995; Roberts *et al.*, 1997a); damping-off due to *Pseudomonas* spp. (Anith, 1997; Barbosa *et al.*, 1995; Gomes *et al.*, 1996; Pal, 1995); root rot of important crops like wheat, cucumber, tulip caused by *Pythium* spp. (Moulin *et al.*, 1994, 1996; Paulitz *et al.*, 1992; Roberts *et al.*, 1997a,b; Weststeijn, 1990); take all disease of wheat (causal organism *Gaeumannomyces graminis* var. *tritici*) (Bull *et al.*, 1991; Duffy *et al.*, 1996; Pierson and Weller, 1994) and several other fungal diseases like charcoal rot (causal organism *Macrophomina* spp.) (Pal, 1995) and grey mould (causal organism *Botrytis cinerea*) (Walker *et al.*, 1996), sorghum anthracnose (Causal organism *Colletotrichum graminicola*) (Michereff *et al.*, 1994a) and seedling blight of cucumber and Poinsettia caused by *Pythium aphanidermatum, P. splendens* and *Rhizoctonia solani* (Wu-Ws, 1995).

The above cited literature clearly indicate the importance of bacterial inoculants in biocontrol of fungal diseases of plants. Inspite of umpteen reports on exploitation of bacterial endophytes as a means for controlling plant diseases, the exact mechanisms by which these microorganisms confer increased plant protection have not been fully investigated.

Numerous hypotheses for protection have been proposed including production of siderophores, accumulation of antifungal metabolites, nutrient competition, and niche exclusion (Chen *et al.*, 1995). Traits of fluorescent

Pseudomonas spp. involved in suppression of plant root pathogens have been critically reviewed by O'Sullivan and O'Gara (1992).

2.7 Solubilization of mineral phosphates

A majority of agricultural soils contain large reserves of phosphorus, of which a considerable part is accumulated as a consequence of regular applications of P-fertilizer (Richardson, 1994). The phenomenon of fixation and precipitation of P in soil is dependent on pH. It can reduce efficiency of soluble P fertilizers (Goldstein, 1986). In acidic soils, P is precipitated as Al and Fe phosphates, whereas in calcareous soils high concentration of Ca results in P precipitation. The soil is indeed a habitat for diverse group of organisms which employ variety of solubilization reactions to release soluble phosphorus from insoluble phosphates (Salih *et al.*, 1989; Illmer and Schinner, 1995; Illmer *et al.*, 1995; Singh and Kapoor, 1998). The potential of these phosphate solubilizing microorganisms (PSM) have been utilised as bioinoculants for crops grown in soils low in available P and amended with rock phosphate or tricalcium phosphate (Salih *et al.*, 1989; Chabot *et al.*, 1993; Chabot *et al.*, 1996; de Freitas *et al.*, 1997).

In growth chamber studies, *Bacillus circulans* and *Bacillus megaterium* var. *phosphaticum* inoculants increased plant weight and P-uptake of millet and pea, respectively (Saber *et al.*, 1997; Raj *et al.*, 1981). Datta *et al.* (1982) found that a P-solubilizing and IAA producing strain of *B. firmus*, increased the grain yield and P-uptake of rice grown in P-deficient soil amended with rock phosphate. Increased biomass and P-uptake was observed in wheat inoculated with *Penicillum bilaji* (Kucey, 1987). He reported a direct correlation between population of P-solubilising *Pencillium bilaji* and wheat dry matter, seed and P yield (Kucey, 1988). Inoculation with *P. bilaji* actually stimulated uptake of Cu, Fe and Zn in wheat plants (Kucey 1988). Gaind and Gaur (1991) reported increased biomass, grain yield, and P and N uptake by mungbean inoculated with *B. subtilis*. Chabot *et al.* (1993) demonstrated that some PSM isolated from Quebec soils stimulated the growth of maize and lettuce in field trials. Several phosphate solubilizing strains belonging to genera *Bacillus* and *Xanthomonas* were found to enhance the growth and yield of canola (*Brassica napus L.*) but did not influence the P-uptake by plant (de Freitas *et al.*, 1997). They suggested the production of IAA-like hormones by these rhizobacteria to influence the growth and yield of plant. Rhizobia have also been shown to solubilize organic phosphates (Haldar and Chakrabarrty, 1993; Abd-Alla 1994; Chabot *et al.*, 1996). Chabot *et al.* (1996) reported the increase in dry matter and P-uptake of maize and lettuce by inoculation of two strains of *Rhizobium leguminosarum* biovar *phaseoli* (P31 and R 1).

Organic phosphorus present in soil must be hydrolysed to inorganic P before it can be utilised by plants (Tate, 1984). Organic P is catalysed through hydrolysis of C-O-P ester bonds by phosphatase or phytase, which are very important in the nutrition of plants (Tarafdar and Claussen, 1988). In phytate acid soils which are rich in organic P, Aspergilli were found to have the highest activity of the fungi tested (Tarafdar *et al.*, 1988). These phosphatase producing *Aspergillus* strains when inoculated in acid soils of Western Rajasthan (India), increased the P uptake and biomass yield of wheat and chickpea (Tarafdar and Rao, 1996). El Sawah *et al.* (1995) reported positive effect of applying phytate biofertiliser on the production of soluble phosphates, plant growth, P and N uptake by maize (cv Giza 2). However, plant growth and nutritional stimulation were not observed following inoculation of *Pueraria phaseoloides* with phytate-mineralizing bacteria (Toro *et al.,* 1996).

2.8 Effect of PGPR on plant growth and yield

There is an enormous volume of literature on the application of bacteria for improvement of plant performance (Whipps and McQuilken 1993; Okon and Labandera-Gonzalez, 1994a; Bashan and Holguin, 1997; Okon *et al.,* 1998; Saxena and Tilak, 1998; Saxena *et al.,* 2000), but few bacteria like *Azotobacter* and *Azospirillum* have been developed as commercial products. The organisms under most scrutiny for potential use in agriculture are bacteria belonging to the genera *Pseudomonas* and *Bacillus* species (Powell and Rhodes, 1994).

The effects of PGPR on crop growth have been evaluated by a number of workers (Burr *et al.,* 1978; Suslow and Schroth 1982; Turner, 1987; Yahalom *et al.,* 1988; Bolton *et al.,* 1990; de Freitas and Germida 1990; Malik *et al.,* 1995; Dashti *et al.,* 1997; Sturz *et al.,* 1997; Kozyrovska *et al.,* 1997; Chiarini *et al.,* 1998). Inoculation of three PGPR isolates each belonging to *Proteus vulgaris, Klebsiella planticola* and *Bacillus subtilis* increased the seed yield of sunflower cv. OSH 1007 from 49-62% over uninoculated plants grown in field (Malik *et al.,* 1995). Fages and Arsac (1991) also reported increase in yield of sunflower due to inoculation of two *Azospirillum lipoferum* strains and one *Xanthomonas maltophila* strain. Increase in plant height and root and shoot biomass of wheat was reported following inoculation with 12 different isolates of PGPR belonging to *Pseudomonas aeruginosa, P. cepacia, P. fluorescens* and *P. putida* (de Freitas and Germida 1990). Similarly treatment of wheat seeds with fluorescent pseudomonads (antagonistic to *Gaeumannomyces graminis*) resulted in yield increases of 27% in field trials (Weller and Cook, 1986). Three PGPR strains namely, *Burkholderia cepacia* strain PHP7, *Pseudomonas fluorescens* strain A 23/T3c and *Enterobacter* sp. strain BB23/T4d were tested in combination or alone for growth promotion of *Sorghum bicolor*. All three strains were able to colonize the root system of sorghum but only *B. cepacia* and *P. fluorescens* promoted plant growth in single strain inoculation tests. Dual strain inocula were no more effective than single ones (Chiarini *et al.*, 1998).

Malik *et al.* (1997) observed IAA production in five strains of PGPR. These PGPR when inoculated in kallar grass and rice resulted in increased acetylene reducing activity. But no correlation was found between IAA production and ARA. Glick *et al.* (1997) studied the effect of a wild type *Pseudomonas putida* GR 12-2 and a mutant *P. putida* GR 12-2/acd 68 which lacked ACC deaminase activity which hydrolyse ACC, the immediate precursor for the phytohormone ethylene (ethylene inhibits root growth). It was observed that wild type, but not the mutant strain, promoted root growth of canola. Lazarovits and Nowak (1997) observed a 60% increase in cytokinin contents of potato plant when inoculated with *Pseudomonas* strain PsJN.

Experiments performed at Chernobyl showed that coinoculation of 'duet' of nitrogen fixing *Klebsiella oxytoca* VN 13 and *Xanthomonas maltophila* VN 12 could protect maize from radionuclides penetration; as well improve the yield and percentage of protein in seed (Kozyrovska *et al.*, 1997).

Malik *et al.* (1999) reported significant increase in the growth and yield of rapeseed due to inoculation of *Kurthia* sp., *Proteus vulgaris, Klebsiella planticola* and *Bacillus subtilis.*

Of the 121 isolates tested with the green gram cv Pusa 105, sixteen strains (3 of *Pseudomonas* spp., 9 of *Bacillus* spp. and 4 of *Enterobacter* spp.) improved plant growth by more than 60% relative to the control (Gupta *et al.,* 1998b).

de Freitas *et al.* (1997) screened phosphate solubilizing bacteria for their ability to enhance the growth and phosphorus uptake of canola. Although some of the P-solubilizing rhizobacteria increased plant height or pod yield, none increased P-uptake. Their results demonstrate the potential use of these P-solubilizing rhizobacteria as inoculants for canola, but indicate that P-solubilization was not the main mechanisms responsible for positive growth response.

Rhizobium, a symbiotic nitrogen fixer, have been shown to act as a plant growth promoting rhizobacteria for non-leguminous crops. Reports indicate that non-legumes react to the presence of bradyrhizobia and rhizobia in the rhizosphere. Root hair curling induced by these symbiotic bacteria was observed on maize, rice and oat plants (Plazinsky *et al.,* 1985; Terouchi and Syono 1990). Studies also show that Nod-factors produced by *Bradyrhizobium* and *Rhizobium,* can be perceived by tomato, as indicated by the induction of alkalization in tomato cell cultures. Nodulating and non-nodulating strains of *Rhizobium leguminosarum* produce IAA (Wang *et al.,* 1982). Noel *et al.,* (1996) observed under gnotobiotic conditions, a direct growth promotion of the early seedling root of canola and lettuce by *R. leguminosorum.* Chabot *et al.* (1996a) observed that phosphate solubilizing strains of *R. leguminosarum* bv *phaseoli* improved the growth of maize and lettuce. Hotlich *et al.* (1994) obtained significant shoot dry matter yield increases (7-8%) by inoculating maize, spring wheat and spring barley (*Hordeum vulgare* L.) with strain R 39 of *R. leguminosarum* bv. *trifolii,* in field experiments. Yanni *et al.* (1995) also observed that certain effective wild type strains of *R. leguminosarum* bv. *trifolii* establish natural plant-bacterial associations that have the potential to promote rice growth under both field and laboratory conditions. Antoun *et al.*, (1998) also observed increase in the dry matter yield of radish due to inoculation of strain Tal 629 of *B. japonicum.*

Coinoculation of PGPR strains with the root nodule bacteria (*Rhizobium* and *Bradyrhizobium*) has been shown to influence the nodulation, nitrogen fixation and growth of leguminous plants. Grimes and Mount (1987) found that *Pseudomonas putida* strain M17 increased *Rhizobium* nodulation of bean in field soils. In Soybean, inoculation of a strain of *P. fluorescens* can enhance the nodulation ability of *B. japonicum.* Preincubation of *B. japonicum* with *P. fluorescens* before inoculation further increased the level of nodulation (Nishijima *et al.*, 1988). Chanway *et al.* (1989) tested nine PGPR strains on a single cultivar of lentil and pea in the field. None of the strains stimulated the growth of pea, but in plots inoculated with one or more rhizobacterial strains, there were significant increase in emergence, vigour, nodulation; C_2H_2 reduction activity and root weight. Bolton *et al.* (1990) found that preinoculation of *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* increased the level of nodulation in peaplant indicating a bacterial-bacterial interaction. Turner and Backman (1991) observed that treatment of peanut seeds with *Bacillus subtilis* was associated with improved germination and emergence, enhanced nodulation by *Rhizobium* spp., enhanced plant nutrition, reduced levels of root cankers caused by *Rhizoctonia solani* AG-4 and increased root growth. Singh (1994) suggested the role of phosphate solubilising microorganisms in stimulating the activity of native rhizobial strains resulting in significant increase in nodulation, plant growth and grain yield (0-41.38%) in soybean plants. Podile (1995) found that seed bacterization of pigeonpea with *Bacillus subtilis* AF1 enhanced the percentage emergence, growth and nodulation.

Singh and Gaur (1992) reported two strains of rhizospheric bacteria that further improved the nodulation as well as competitiveness of an effective strain of chickpea *Rhizobium*. They further suggested the role of flavonoid like substances produced by rhizobacterial strains in nod gene expression (Singh and Gaur, 1995). Similar results were obtained by Parmar and Dadarwal (1999) in case of chickpea. Coinoculation of the rhizobacteria with effective *Rhizobium* strains of chickpea resulted in a significant increase in nodule weight, root and shoot biomass and total plant nitrogen in chickpea. The nodule stimulating rhizobacteria enhanced levels of flavonoid-like compounds in roots on seed bacterization. Also, ethyl acetate extracts of culture supernatant fluids when applied to seeds resulted in enhancement of flavonoids in roots, suggesting that the rhizobaceria have a direct influence on root flavonoids which might be an additional factor in root promotion by these bacteria (Parmar and Dadarwal, 1999).

IAA producing *Bacillus* isolates promoted root growth and (or) nodulation when coinoculated with *Rhizobium etli* on *Phaseolus vulgaris* 'contender' in growth chambers (Srinivasan *et al.*, 1996). Similarly coinoculation of soybean with *B. japonicum* and *Serratia liquefaciens* 2-68 or *S. proteamaculans* 1-102 increased soybean grain and protein yield compared to the non treated controls (Dashti *et al.*, 1997, 1998). Gupta *et al.* (1998) isolated 16 strains of PGPR from rhizotic zone of green gram which were identified as *Enterobacter, Pseudomonas* and *Bacillus* spp. These PGPR were found to increase shoot and root length and fresh and dry biomass of mungbean plants. *Enterobacter* isolate EG-ER-2 increased the nodule occupancy of *Bradryhizobium* spp. strain S24 on mungbean and another isolate KG-ER-1 did the same for rhizobial strain COG 15 (Gupta *et al.*, 1998).

Gupta *et al.* (1998) observed 60-80% increase in nodule occupancy and increased shoot biomass, N-contents and grain yield of mungbean plant as a result of coinoculation of PGPR and *Bradyrhizobium*.

Beneficial effects of coinoculation of *Azospirillum* with *Rhizobium* have been reported by different workers (Tilak *et al.*, 1981; Sarig *et al.*, 1986; Yahalom *et al.*, 1987; Saxena and Tilak, 1994; Galal, 1997; Tchebotar *et al.*, 1998). Coinoculation of *Rhizobium* spp. and *Azospirillum* spp. enhances the nodule number and grain yield of various leguminous crops (Sarig *et al.*, 1986; Yahalom *et al.*, 1987). Coinoculation also increases the shoot length, dry weight, number of root hairs and root diameter of alfalfa (Itzigsohn *et al.*, 1983). Dual inoculation with a mixture of *Bradyrhizobium japonicum* and *A. brasilense* strains improved growth and nitrogen fixation of soybean (Galal, 1997). The combined inoculation of white clover with *Rhizobium leguminosarum* bv. *trifolii* and *A. lipoferum* enhanced the number of nodules by 2-3 times and acetylene reduction activity by 2.3-2.7 times (Tchebotar *et al.*, 1998).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

Seeds of wheat (*Triticum aestivum*) cv. HD 2285, mustard (*Brassica juncea*) cv. Pusa Bold, sunflower (*Helianthus annus*) cv. Pusa 105, chickpea (*Cicer arietinum*) cv. Pusa 256, lentil (*Lens culinaris*) cv. L 4147, soybean (*Glycine max*) cv. Pusa 22 and mungbean (*Vigna radiata*) cv. Pusa 9072 were obtained from Divisions of Genetics and Agronomy, IARI, New Delhi. Teff (*Evagrostis tef*) cv. DZ-01-354 seeds were obtained from Holetta Research Centre, Ethiopia.

3.1.2 Fertilizers

Urea and single super phosphate (SSP) were used as a source of N and P respectively and were obtained from the Division of Agronomy, IARI, New Delhi.

3.1.3 Microbial cultures

(a) ***Bacterial cultures*** : Bacterial cultures *Bacillus subtilis* (Bs), *Klebseilla planticola* (Kp), *Proteus vulgaris* (Pv), *Bacillus polymyxa, Azospirillum brasilense* and *Azotobacter chrooccocum* were obtained from culture collection, Division of Microbiology, IARI, New Delhi.

(b) ***Fungal cultures*** : Fungal cultures *Colletotrichum falcatum, Fusarium moniliforme, Rhizoctonia solani, Pythium aphanidermatum, Macrophomina phaseolina* and *Fusarium solani* were obtained from the

3.1.4 Growth media used in the study

Different media were used for growth of the organisms. All constituents of the media are given in gram per litre distilled water until and unless mentioned.

(a) Nutrient Agar (Cappuccino and Sherman, 1992)

Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	18.0
pH	7.0

(b) Jensen's media (Subba Rao, 1995)

Sucrose	20.0
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.5
NaCl	0.5
$FeSO_4.7H_2O$	0.1
$CaCO_3$	2.0
Agar	18.0
pH	7.0

(c) Nitrogen free bromothymol blue sodium malate medium (Bulow and Doberiener, 1975)

Malic acid	5.0
KOH	4.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.05
$MnSO_4.H_2O$	0.01
NaCl	0.02
$Na_2MoO_4.2H_2O$	0.002
BTB (0.5 % alcohol solution)	2.0 ml
pH	6.5

NH_4Cl was added for solid medium @ 1 gl^{-1}. For semi-solid medium 0.18% and solid medium 1.8% agar was used.

(d) Pikovskaya agar medium (modified by Sundara Rao and Sinha, 1963)

Glucose	10.0
Tricalcium phosphate	5.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4.7H_2O$	0.1
$MnSO_4$	Trace
$FeSO_4.7H_2O$	Trace
Yeast extract	0.5
Agar	18.0
pH	7.0

(e) Tryptone medium (Cappuccino and Sherman, 1992)

Tryptone	10.0
Sodium chloride	5.0
Calcium chloride (1M)	1.0 ml
Agar	11.0

(f) Basal medium (Peptone water) (Cappuccino and Sherman, 1992)

Peptone	10.0
NaCl	5.0
pH	7.0
BCP	20.0 ml

(g) Chrome Azurol S (CAS) medium (Schwyn and Neilands, 1987)

Chrome Azurol S blue agar medium was modified by substituting nitrogen containing sodium malate medium for the MM9 salts medium.

Solution A

To prepare 1 litre of CAS medium 60.5 mg Chrome Azurol S (CAS) was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). This solution was slowly added to 72.9 mg Hexadecyltrimethylammonium bromide (HDTMA) dissolved in 40

Solution B

Deferrated 1M malic acid	3.5 ml
Deferrated 1M $CaCl_2$	0.4 ml
Deferrated 1M $MgSO_4.7H_2O$	0.8 ml
Deferrated 2% K_2HPO_4	10.0 ml
Deferrated 2% NaCl	10.0 ml
Deferrated 10% NH_4Cl	10.0 ml
Deferrated 1% $NaMoO_4$	0.5 ml
PIPES (1,4-piperazine di ethane sulfonic acid)	30.24 g
Difco agar	15.0 gm
Deferrated double distilled water	Volume made upto 800 ml

The pH of the medium was adjusted to 6.8 by the addition of 50% (w/w) NaOH (12 gm) before autoclaving. After cooling to 50°C, 30 ml of deferrated filter sterilized casamino acids (10%) was added.

The solution A was finally added to solution B along the glass wall with enough agitation to achieve maximum mixing without formation of foam.

Deferration

In siderophore studies contaminating iron should be avoided to get good results, so treating the chemicals and glassware for removal of iron, therefore, becomes essential (Cox, 1994).

Removal of contaminating iron from glassware

Glass is a good ion exchange surface and hence may get contaminated with iron on surface. All glassware used for siderophore study are soaked in 2 N HCl for 24 hrs and washed with double distilled water to remove the acid.

Removal of contaminating iron from media components

Media components such as malate, K_2HPO_4, $MgSO_4$ etc. were deferrated by extraction with 3% (w/w) 8-hydroxyquinoline in chloroform twice. The residual qunioloine if any, was removed by extraction with pure chloroform (Schwyn and Neilands, 1987).

(h) Potato dextrose agar medium (PDA) (Subba Rao, 2000)

Chemicals	g l^{-1} medium
*Potato extract	450 ml
Distilled water	450 ml
Dextrose	9.0
Peptone	1.0
Agar	16.0

*Potato extract : 250 g peeled and sliced potato were boiled in 1 l of distilled water for 1 hr and filtered through a muslin cloth and the volume was made to 1 l.

(i) Simmons citrate agar (Cappiccino and Sherman, 1992)

Chemicals	g l^{-1}
Sodium citrate	5.0
NaCl	5.0
$NH_2H_2PO_4$	1.0
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.2
Agar	16.0
Bromothymol blue	0.08
pH	6.9

(j) Starch agar (Cappuccino and Sherman, 1992)

Chemicals	g l^{-1}
Peptone	5.0

Starch (soluble)	2.0
Agar	16.0
pH	7.0

(k) Nitrate broth (IARI, 1995)

Peptone	10.0
Beef extract	5.0
KNO_3 (nitrite free C.P.)	1.0
Distilled water	1000.0 ml

(l) H_2S test (Modified from IARI, 1995)

Peptone	10.0
Beef extract	10.0
NaCl	5.0
Cysteine	0.1
pH	7.1

3.1.5 Reagents

(a) Griess-Ilosvay's reagent (Pelczar *et al.*, 1957)

Solution A	$g\ l^{-1}$
Sulphanilic acid	8.0
Acetic acid (5 N)	1000.0 ml
Solution B	
Napthylamine	5.0
Acetic acid (5 N)	1000.0 ml

Mixed solutions A and B in equal proportion.

(b) Iodine solution for starch hydrolysis (Lugol's iodine) (Seedey and Van Demark, 1980)

Iodine	50.0
KI	100.0

(c) Nessler's reagent (Cappuccino and Sherman, 1992)

KI	50.0
Distilled water (ammonium free)	35.0

Add saturated aqueous solution of mercuric chloride ($HgCl_2$) until a slight precipitate persists.

Potassium hydroxide (50% aqueous)	400.0 ml

Diluted to 1000 ml with ammonium free distilled water. Allowed to stand for 1 week in refrigerator decanted the supernatant and stored in a tightly capped amber bottle.

(d) Salkowski's reagent (Gorden and Weber, 1950)

0.5 M $FeCl_3$	1.0 ml
35% $HClO_4$	50.0 ml

(e) Kovac's reagent (Bailey and Scott, 1960)

p-dimethyl amino benzaldehyde	10.0
Amyl alcohol	150.0 ml
HCl (conc.)	25.0 ml

The p-dimethyl amino benzaldehyde was dissolved in the amyl alcohol, HCl was then added and kept in a glass stopper bottle and was stored in refrigerator.

(f) Bradford reagent (Bradford, 1976)

BSA	0.5 mg ml^{-1}
Comassive Brilliant blue	100.0 mg

Comassive brilliant blue solution : In a one litre volumetric flask 100 mg of Comassive brilliant blue G-250 was dissolved in 50 ml of 95% ethanol. To it, 100 ml of 85% phosphoric acid was added. Final volume was made to one litre. Contents were filtered through Whatman no. 1 filter paper. Filtrate was stored at 4°C.

(g) Reagents for P-solubilization

a : $SnCl_2$ — 2.5 g in 10 ml of concentrated HCl and volume is made 100 ml with distilled H_2O.

b : NH_4MoO_4 — 15 g in 300 ml H_2O warmed and 342 ml conc. HCl was added and volume was made to 1 L with distilled water.

Ammonium molybdate

Ammonium vandate in HNO_3 was prepared by dissolving 22.5 g $(NH_4)_6\ Mg_7O_{24}.4H_2O$ in 400 ml distilled water. Dissolve 1.25 g ammonium vandate was dissolved in 300 ml boiling distilled water. The vandate solution was added to the molybdate solution and cooled to room temperature. 250 ml of concentrated HNO_3 was added and diluted to one litre.

Phosphate standard solution

Analytical grade KH_2PO_4 (0.2195 g) was dissolved and diluted to one litre. This solution contains 50 Ng P ml^{-1} (50 ppm).

3.2 Methods

3.2.1 Sterilization

The glasswares were dry sterilized in a hot air oven at 180°C for

(1.1 kg cm^{-2}) pressure at 121°C for 30 minutes. Heat labile chemicals, antibiotics and sugars were filter sterilized using 0.45 µm membrane filters.

3.2.2 Incubation

All the bacterial cultures of *Bacillus subtilis, B. polymyxa, Klebsiella planticola* and *Azospirillum* were incubated at 37°C whereas *Rhizobium*, *Azotobacter* and *Proteus vulgaris* were incubated at 28°C for 2-3 days for growth. The broth cultures were kept in rotary shaker at 150 rpm.

3.2.3 Cultural and Biochemical Characterization of PGPR Strains

Cultures of *Bacillus subtilis, Klebsiella planticola* and *Proteus vulgaris* were characterized biochemically for production of siderophore, IAA, solubilization of phosphates, resistance to different antibiotics, carbohydrate utilization, nitrate reduction, H_2S production and citrate utilization.

(a) *Growth and colony characterization* : A loopful of young broth culture was suspended in the dispersion fluid and streaked over nutrient agar plates to obtain discrete colonies. After required incubation period, colony characteristics (like colour, shape, margin, elevation and opacity) were recorded.

(b) *Utilization of carbohydrates* : Best growth media for each PGPR was used to study the comparative utilization of different sources of sugars and other carbon sources. The medium used for *B. subtilis* was Pikovskaya Agar for *P. vulgaris* was Jensen's media and *K. planticola* was Nitrogen Free BTB. These include glucose, galactose, dextrose, sucrose, lactose, maltose, mannitol, dulicitol arabinose, xyleose etc. Bacterial cultures were

(c) ***Siderophore production*** : Siderophore production was examined by following the universal assay method of Schwyn and Neilands (1987). Cultures were spot-inoculated and incubated at 30ºC for 7 days. Development of orange zone around the bacterial growth was considered to be a positive test for the production of siderophore.

(d) ***Intrinsic antibiotic resistance (IAR) pattern of rhizobacterial species*** : The IAR pattern was studied following the procedure described by Josey *et al.* (1979). The eight antibiotics used for the study and the solvents used for preparing them are listed in Table 1.

Stock solutions (10 mg/ml) of the antibiotics were prepared in the appropriate solvent (Table 1), filter sterilized and stored at 4ºC. Agar based medium was prepared by dispensing 50 ml in 100 ml conical flasks and autoclaved at 15 lbs psi. Medium was cooled to 50-60ºC and appropriate

Table 1 Antibiotics and their solvents

Antibiotics	Solvents
Ampicilin	Distilled water
Chloramphenicol	Methanol
Kanamycin	Distilled water
Nalidixic acid	Distilled water + NaOH
Spectromycin	Distilled water
Streptomycin	Distilled water
Trimitheporin	Distilled water

concentration of antibiotic was added. Ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin and trimethoprin were added at concentrations 100, 125, 150 and 200 µg ml^{-1} whereas tetracycline was tested at 5, 10, 15 and 25 µg ml^{-1}. Two plates were pooled for each concentration and allowed to solidify. PGPR strains were spot inoculated on the antibiotic plates and control plates (no antibiotics). Inoculated plates were incubated for 24 hrs at 37°C for first observation and further 24 hrs for next observation. After incubation, the growth on antibiotic plate was compared with control plates. Growth was scored as positive or negative concentration until which the growth occurred was taken as the resistance limit.

(e) ***Production of indole acetic acid*** (Gorden and Walebal, 1951)

(i) ***Quantitative estimation of IAA*** : Six ml tryptone broth containing tubes inoculated with the PGPR isolates were incubated for 72 hrs.

To each culture 3 ml of Kovacs reagent was added. It was mixed well by rotating the tube between the hands and upon standing the alcohol layer was separated from the aqueous layer. A reddening of the alcohol layer within a few minutes indicates that indole is present.

(ii) ***Spectrophotometric method*** : The bacterial cultures were raised in tryptone broth containing tryptophan for 72 hrs and two ml were centrifuged at 10,000 rpm for 20 minutes. The pellets were used for protein estimation. To the supernatant two drops of O-phosphoric acid were added. Then four ml of the $FeCl_3$ reagent solution was added and incubated for about half an hour at room

broth served as control. Auxin concentration values were obtained by preparing standard curve using standard IAA (0-100 µg ml^{-1}) and expressed as µg IAA mg $^{-1}$ protein.

(iii) ***Protein estimation*** : The pellet was used for estimation of protein. Pellet was incubated at 100^{0}C for 30 min with 0.5 ml of 2 N NaOH following incubation, 0.5 ml of 2 N HCl was added to suspension and was used for colorimetric assay of the total proteus according to Bradford (1976).

(iv) ***Standard curve for protein*** : A standard solution of BSA 0.5 mg ml^{-1} was prepared and was added in set of 11 test tubes at a concentration of 0-100 µg ml^{-1} in triplicate. After making volume to 1 ml and then 5 ml of comassie brilliant blue G-250 solution was added under vigorous shaking. It was allowed to stand for 10-15 minutes and then the optical density (OD) was measured at 595 nm (Table 3.2). The values obtained were plotted on graph and used for estimation of protein in unknown samples.

(f) ***Starch hydrolysis activity (Cappuccino and Sherman, 1992)*** : The test cultures were spotted on nutrient agar supplemented with 0.2% starch powder as carbon source and incubated. After 72 hrs the plates were flooded with iodine solution and observed for the clear zone around the growth, an indication of starch hydrolysis.

(g) ***Utilization of citrate (Cappuccino and Sherman, 1992)*** : Simmone citrate agar plants were streaked with the test cultures and incubated for 72 hrs and the observation of change of colour from green to blue was taken as positive.

(h) ***Reduction of nitrate to nitrite (Cappuccino and Sherman, 1992)*** : Five ml basal medium containing 0.2 % KNO_3 was sterilized in test tubes. The test tubes were inoculated with different cultures and incubated. After 72 hrs formation of nitrite was detected by the development of red colour on the addition of Griess-Ilosvay's reagent to the cultures in tubes.

(i) ***Production of ammonia from peptone (Cappuccino and Sherman, 1992)*** : Ammonia is a volatile substance produced by many rhizobacteria, and is toxic to fungi. For the detection of ammonia-production, 5 ml peptone water in each test tube were inoculated and incubated. After 48 hrs, 1 ml of Nessler's reagent was added to each culture tube. Change of colour from yellow to brown indicated the presence of ammonia.

(j) ***Production of hydrogen sulphide (H_2S) (Cappuccino and Sherman, 1992)*** : Test tubes containing peptone water incorporated with beef extract and cystein and hanging filter paper strip saturated with lead acetate were inoculated. After 72 hrs observations were taken for blackening of filter paper strip as positive test.

(k) ***Phosphate solubilization test*** : Phosphate solubilization was tested by inoculating the PGPR on to Pikovskaya agar medium and subsequent incubation of plates. After 2-6 days plates were observed for clearing zones around the bacterial growth.

(l) ***Quantification of P-solubilization test*** : The three PGPR were grown on Pikovskaya's broth medium and were incubated. After 4, 8 and 12 days interval reading was taken for solubilized P. One ml culture

Spinwin microcentrifuge. 0.5 ml of the supernatant was taken and 10 ml of ammonium molybdate was added plus 30 ml distilled H_2O. And 0.5 ml of Stanous chloride solution and volume was made 50 ml. Reading at 600 nm was taken spectrophotometrically.

(m) ***Preparation of P-standard curve*** : A standard solution containing 0, 1,2,3,4 and 5 ml was transferred to 50 ml volumetric flask to get 0, 1,2,3,4 and 5 ppm of P respectively. 10 ml of Vanadomohybdate reagent was added to each flask and the volume was made using deionized water and was shaken thoroughly. Absorbance was recorded after 30 minutes of incubation at 600 nm using spectrophotometer with blue filter. Sample values were obtained using the standard curve.

(n) ***Nitrogenase activity of the PGPR*** : The nitrogenase activity of PGPR under cultural conditions was assayed by growing them in combined carbon medium (Rennie, 1981) following the procedure outlined in section 3.2.11

(o) ***Anti-fungal test*** : The PGPR were tested for their ability to suppress fungal growth by growing them on PDA media. The bacterial cultures were streaked on three sides of the media plate while the fungal inoculum disc was put to grow at the centre of the petri plate.

3.2.4 Carrier based inoculant and shelf life

The PGPR cultures were raised in their respective medium.

Carrier based inoculant of PGPR was prepared by using sterilized charcoal : soil (3 : 1) as carrier and following the method described by Jauhri *et al.* (1979).

Carrier was prepared by using charcoal (75%), soil (25%), $CaCO_3$ (0.1%), K_2HPO_4 (0.05%) and water (10%). All these were mixed very well to make a homogenous mixture and filled up in a tray. This was covered with aluminium foil and put in autoclave for sterilization at 121°C (15 psi) for 3-4 hrs. Freshly grown bacterial culture @ 30% was added aseptically to the carrier and were well mixed. The moisture available will be 40% and this was filled in sterilized polythene packets and sealed immediately. One g of the inoculant is serially diluted and plated for viable count of the rhizobacteria to give readings at 0 hr time. The bacterial count was determined at different intervals of time 48 hrs, 15, 30, 60, 90, 120, 150 and 180 days. The moisture content of the carrier was also determined at similar intervals of time.

3.2.6 Seed Inoculation

Sterilized sucrose solution (10%) 40 ml, carrier based inoculum of PGPR strains 50 g and 500 g of seeds were mixed thoroughly in polyethylene bags and the mixture was air dried for 10 minutes under shade. The seeds were sown immediately without exposing to sunlight as it may cause the death of inoculum.

3.2.7 Field Experiments

All field experiments were conducted at the IARI research field with soil type sandy loam having a pH 7.6 with 172.2 kg/ha availabile N, 13.8 kg P_2O_5 and 182 kg K_2O/ha and organic carbon 0.405.

(a) ***Response of wheat to inoculation with PGPR*** : A released variety of wheat HD 2285 was sown during winter (*rabi*) season of 1998/99. The field experiment was executed in a split plot design with four replications. Three nitrogen fertilizer levels were taken as main block. Five PGPR inoculants along with one uninoculated control was taken in sub plots. The experimental details are as follows :

Main plots : N levels

N_0 : No nitrogen
N_1 : 40 kg N ha^{-1}
N_2 : 80 kg N ha^{-1}

Sub plots : PGPR cultures

1. Uninoculated control
2. *Bacillus subtilis*
3. *Klebsiella planticola*
4. *Proteus vulgaris*
5. *Azospirillum* CDJA
6. *Azotobacter* W5

Total number of plots : 72
Plot size : 4 x 5 sq. meters
Row spacing : 30 cm

Five plant samples were harvested from each plot after 45 days of plant growth. The soil closely adhering to the roots were shaken and used to estimate the population of inoculant strains following dilution plate count method. Specific media amended with selective antibiotic was used to enumerate the bacterial population. The roots and shoots were oven dried at 80°C for 3 days and dry weight was recorded.

At harvest, the plant height of the standing crop was measured. Straw and grain yield was also recorded.

(b) *Response of soybean to inoculation with PGPR* : Soybean cultivar Pusa 22 seeds were sown during *kharif* season of 1999 in furrows after being dressed with respective PGPR inoculants. The details of the

Crop : Soybean

Cultivar : Pusa 22

Design : Randomized block design

Treatment : 1. Control
2. *Bacillus subtilis* (Bs)
3. *Klebsiella planticola* (Kp)
4. *Proteus vulgaris* (Pv)
5. *Bradyrhizobium japonicum* (SB 271)
6. SB 271 + Bs
7. SB 271 + Kp
8. SB 271 + Pv
9. 80 Kg N ha^{-1}

Replications : Three

Plot size : 4 x 2 sq mt.

Row spacing : 30 cm

A pre-sowing irrigation was given 10 days before field preparation. Single super phosphate @ 80 kg ha^{-1} was applied during field preparqation.

(c) ***Response of lentil to inoculation with PGPR*** : A field trial was conducted in the *Rabi* of 1998-99 using lentil cv. L 4147. The details of the experiment are as follows :

Crop : Lentil

Cultivar : L-4147

Design : Randomized block design

Treatment : 1. Control

2. *Bacillus subtilis* (Bs)

3. *Klebsiella planticola* (Kp)

4. *Proteus vulgaris* (Pv)

5. *Rhizobium leguminosarum* (L-12-89)

6. Bs + L-12-89

7. Kp + L-12-89

8. Pv + L-12-89

Replications : Three

Plot size : 2 x 2 sq. metre

Row spacing : 30 cm

A presowing irrigation was given 10 days before field preparation. Single super phosphate @ 100 kg ha^{-1} was applied during field preparation.

Five plants were harvested from each plot after 45 days of plant growth of ~~N~~nitrogenase activity in the root nodule system was measured by C_2H_2 reduction assay with gas chromatography as described in section 3.2.10. The data on nodule number, nodule fresh weight, root and shoot dry weight was also recorded. At maturity data on straw and grain yield was also recorded.

(d) *Response of mungbean to inoculation with PGPR* : A field trial was conducted during the *kharif* of 1999 using mungbean cv. Pusa 9072. The experimental details are the same as for lentil experiment except for the use of mungbean specific *Bradyrhizobium* spp. strain M11 and the plot size was 4 x 2 sq. metres.

After 40 days of plant growth, five plants per plot were harvested and the observations made were the same as for lentil field experiment.

(e) ***Response of chickpea to inoculation with PGPR*** : A field trial was conducted during the *Rabi* of 1999-2000 using a chickpea cultivar Pusa 256. The experimental details are same as for lentil experiment except for the use of chickpea specific *Mesorhizobium ciceri* strain F75 and the plot size was 4 x 2 sq. metres.

Data on crop nodulation was recorded 50 days after germination. five plants were randomly selected from each plot to determine nodule number, fresh weight of nodules, dry weight of root and shoot. However, data on straw and grain yield were recorded only after the crop was harvested.

3.2.8 Green House Experiments

The influence of PGPR isolates *Klebsiella planticola, Bascillus subtilis* and *Proteus vulgaris* was studied on mustard, sunflower and teff (*Eragrostis tef*) crop. The experiments were conducted in green house using 10 kg capacity earthen pots. In addition to the three PGPR isolates, biofertilizer strains already in use for each crop were also included for reference. Inoculation was done by applying 10 ml of broth culture (10^8 cells ml^{-1}) in each pot.

(a) ***Response of mustard to inoculation with PGPR*** : Earthen pots were filled with 10 kg soil and phosphorus was applied as basal dressing @ 60 kg P_2O_5 ha^{-1}. Mustard variety Pusa Bold was used for the pot experiment. Two levels of nitrogen were applied (0 and 30 kg Nha^{-1}) and a total of six treatments were maintained. These included : 1.

Uninoculated control, 2. *B. subtilis,* 3. *K. planticola*, 4. *P. vulgaris*, 5. *Azotobacter chroococcum* (M4) and 6. *B. polymyxa.* Each treatment was replicated six times. After 45 days, three replicates for each treatment were harvested and observations on root and shoot dry weight were recorded. The soil adhering to roots was used to enumerate the population of inoculant strains using specific media and selective antibiotic for each isolate. Other three replications were maintained till maturity and observations on grain and straw yield was recorded.

(b) ***Response of sunflower to inoculation with PGPR*** : The pots were prepared as for mustard experiment. Phosphorus was applied @ 60 kg P_2O_5 ha^{-1} before sowing as basal dressing. Sunflower cultivar Mordan was used for the pot experiment. Two levels of nitrogen (40 and 80 kg N ha^{-1}) were applied. The treatments included uninoculated control, *B. subtilis, K. planticola, P. vulgaris* and *Azospirillum.* Each treatment was replicated six times. At maturity, the crop was harvested and observations were recorded on root and shoot dry weight, head diameter and seed yield.

(c) ***Response of Teff to PGPR inoculation*** : The earthen pots were prepared as for mustard experiment. Teff cultivar D-01-354 was tried in pot trial using two levels of N (0 and 46 kg N/ha). PGPR inoculations were done both in the absence and presence of nitrogen fertilizer. The treatments included uninoculated control, *B. subtilis, K. planticola, P. vulgaris, Azospirillum brasilense and Azotobacter chroococcum.* Each treatment was replicated 6 times. The plants were harvested at marutity and observations were made on plant height, head size, straw and grain yield.

3.2.9 Establishment Studies

Rhizospheric soil was collected and air-dried. One g of the soil was serially diluted and plated on medium containing appropriate antibiotic and the count was taken after growth.

3.2.10 Nitrogenase Assay in Nodules

Five healthy plants were randomly but carefully uprooted after plants reach initial flowering stage. The root portion with intact nodules was excised. Excess soil from roots was removed by gentle washing and dried by absorbing water using filter paper. The roots were put in 500 ml bottle which was made air tight with rubber stoppers were then incubated at 28°C temperature for 30 minutes and the gas phase was analysed for acetylene reduction using a Shimadzu Gas Chromatograph GC-14A fitted with flame ionization detector and Porapak R column. The column temperature was kept at 110°C, N_2 was used as a carrier gas with flow rate of 35 ml min^{-1}. One ml gas sample was injected into gas chromatograph and the peak height of ethylene was measured.

Standard ethylene gas (105 VPM) was also injected, peak height measured and used for calculating acetylene reduced to ethylene in samples. The nitrogenase activity was expressed as nmoles of C_2H_4 produced per hr per fresh weight of nodules and calculation was done using the following formula :

$$\text{n moles of } C_2H_4 \text{ produced/hr/g protein} = \frac{C \times Ps \times As \times V}{P_{std} \times A_{std} \times T \times W}$$

where,

C = Concentration of ethylene in the standard in nmoles

Ps = Peak height of sample

As = Attenuation used for sample
P_{std} = Peak height of standard
A_{std} = Attenuation used for standard
T = Incubation time in hrs
W = Weight of nodules (g)
V = Volume of air space in assay vial (ml).

3.2.11 *In vitro Nitrogenase assay of PGPR isolates*

PGPR isolates were grown in tubes contaiing Rennies combined carbon medium for 24 h following incubation, the cotton plugs were replaced with subaseals. Afer sealing 2 ml of air from tubes was replaced with 2 ml of acetylene to make 10% of acetylene atmosphere and further incubated for 24 h at 30°C. The nitrogenase activity was determined after 24 h by method outlined in section 3.2.10. The nitrogenease activity was expressed as nmoles C_2H_4 produced h^{-1} mg^{-1} protein and calculated by the following formula :

$$\text{n moles of } C_2H_4 \text{ produced } hr^{-1}\ mg^{-1} \text{ protein} = \frac{C \times Ps \times As \times V}{P_{std} \times A_{std} \times T \times P}$$

where,

C = Concentration of ethylene in the standard in nmoles
Ps = Peak height of sample
As = Attenuation used for sample
P_{std} = Peak height of standard
A_{std} = Attenuation used for standard
T = Incubation time in hrs
P = Protein content of bacterial growth in tubes in mg
V = Volume of air space in assay vial (ml).

3.2.12 Nitrogen Estimation

Total N in shoots were estimated after drying them in oven at 70-80°C. The dried shoots were ground and 100 mg of sample was digested with sulphuric acid and digestion mixture ($CuSO_4$: K_2SO_4, 1 : 10) at 400°C. The digested samples were analyzed for nitrogen content using Kjeltec autoanalyzer (Tecator Kjeltec 1030 autoanalyzer).

3.2.13 Dry Matter Yield and Seed Yield

Dry matter yield for roots, shoots and straws were recorded from each and every experiment. And seed yield of the crops was taken at final harvest.

3.2.14 Statistical Analysis of the Data

Statistical analysis of data obtained from laboratory, pot and field experiments was done by using different experimental designs (Fisher, 1958). Standard error (Sem ±) and critical difference (CD) at 5% significance level was calculated.

4.0 RESULTS

4.1 Cultural Characteristics

Three PGPR isolates namely *Proteus vulgaris, Klebsiella planticola* and *Bacillus subtilis* were grown on four different media to study their morphological and cultural characteristics. The media employed were nitrogen-free malate medium with bromothymol blue (BTB), Pikovskaya medium, nutrient agar and sodium succinate medium. The growth characteristics in different media is given in Table 2 and shown in Plates 1 and 2.

4.2 Biochemical Characteristics

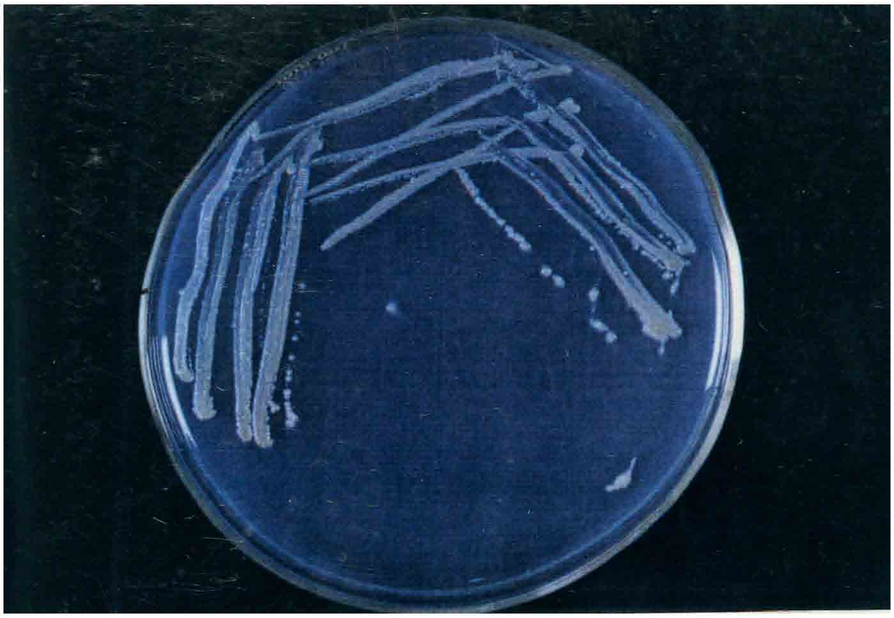
4.2.1 Utilization of Carbohydrates

All the three PGPR isolates were tested for utilization of different sugars in medium. The utilization pattern of various sugars by isolates is show in Table 3. *Bacillus subtilis* isolate was able to grow on all the sugars tested except on mannitol and the growth was less in plates containing dulcitol and sodium succinate. *Klebsiella planticola* could also grow luxuriantly on all C sources tested except for lactose and malic acid. *Proteus vulgaris* was also not able to utilize lactose and could metabolize all other C sources tested. However, the growth was retarded in media containing sucrose, maltose, raffinose, rhamnose, trehalose and dulcitol. On plates containing Na-succinate, *P. vulgaris* showed prolific growth producing brilliant yellow ring around the centre of the colony.

Table 2 Growth characteristics of PGPR isolates on different growth media

Media	*Bacillus subtilis*	*Klebsiella planticola*	*Proteus vulgaris*
1. Nitrogen free malate medium +BTB			
a. colony colour	Light yellow	Yellowish	yellow
b. ring formation	interior ring of yellow colour	Orange yellow at the corners	internal ring away from the centre
c. colour reaction	green to yellow	green to dark blue	green to light blue
d. shape	circular, raised, regular	circular, raised regular	regular
e. margin	entire margin	entire margin	-
2. Pikovskaya medium			
a. colour	white	creamish or dirty white	yellow
b. Growth	profuse (4.8 cm dia in 7-10 days)	small colonies (2.0 cm dia in 7-10 days)	-
c. reaction	clearing zone around growth	clearing zone around growth	insignificant clearing zone around growth
3. Nutrient agar			
a. colour	White	creamy white	dark creamy
b. margin	entire	entire	irregular
c. elevation	raised	raised	thick and raised
4. Sodium succinate			
a. growth	Poor	Good	Luxuriant
b. colour	white	orange creamy with shallow centre	yellow thick ring with clear centre
c. margin	entire	entire	regular

Plate 1. Growth of three PGPR isolates on nitrogen-free malate medium with Bromothymol blue. From top to bottom : *Proteus vulgaris; Klebsiella planticola* and *Bacillus subtilis*



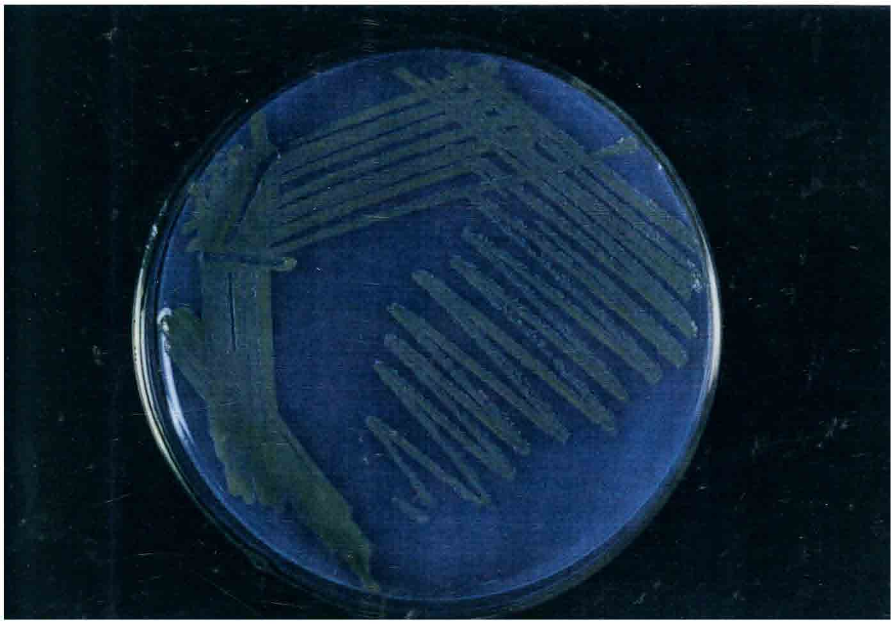
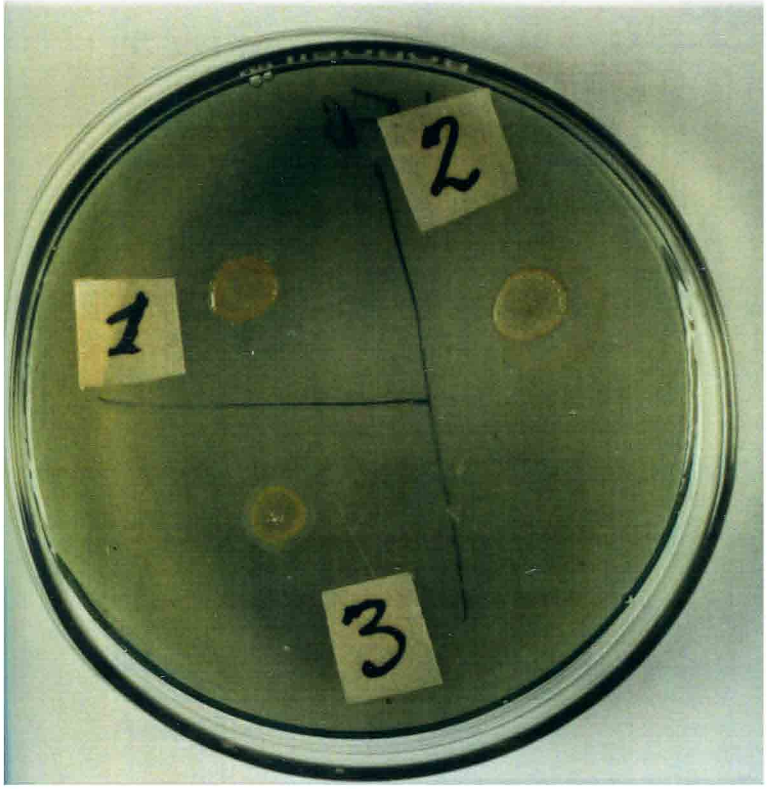


Plate 2. Spot inoculation of three PGPR isolates on nitrogen-f
malate medium with Bromothymol blue

1. *Bacillus subtilis*
2. *Klebsiella planticola*
3. *Proteus vulgaris*



1
2
3

Table 3. Utilization pattern of different carbon sources by PGPR isolates (After 7 days)

	C-source	Organism		
		B. subtilis	*K. planticola*	*P. vulgaris*
1.	Glucose	+++	+++	+++
2.	Galactose	+++	+++	+++
3.	Dextrose	+++	++	+++
4.	Sucrose	+++	++	++
5.	Fructose	+++	+++	+++
6.	Maltose	+++	+++	++
7.	Lactose	+++	–	–
8.	Mannitol	–	+++	+++
9.	Arabinose	+++	+++	+++
10.	Raffimose	+++	+++	+
11.	Rhamnose	+++	+++	+
12.	Trehelase	+	+++	++
13.	Dulcitol	+	+++	++
14.	Xylose	+++	+++	+++
15.	Maleic acid	+++	–	+++
16.	Sodium succinate		+++	+++

+++ = Very good growth
++ = Good growth
\+ = Fair growth
– = No growth

4.2.2 *Intrinsic Antibiotic Resistance Profile*

The intrinsic levels of resistance to antibiotics chloramphenicol, kanamycin, streptomycin, ampicillin, trimethoprin, tetracycline and nalidixic acid was studied and the resistance pattern is shown in Table 4. All the PGPR isolates were found to resist kanamycin, streptomycin and ampicillin till the concentration of 200 µg ml. Of the three isolates, *Bacillus subtilis* showed maximum resistance (200 µg ml^{-1}) to all the antibiotics tested. *Klebsiella planticola* and *Proteus vulgaris* could grow only upto 50 µg ml^{-1} chloramphenicol. The two can be further differentiated from each other in their resistance to trimethoprin, nalidixic acid and tetracycline (Table 4).

4.2.3 *IAA Production*

IAA production by three isolates was tested both qualitatively and quantitatively. Addition of Kovac's reagent showed the formation of cherry red colour ring only in case of *Proteus vulgaris.* Slight colour change was observed in case of *K. planticola* and *B. subtilis*. Quantitative analysis revealed that *P. vulgaris* could produce 153 µg IAA/mg protein (Table 5).

4.2.4 *Siderophore Production*

Three PGPR isolates were tested for the production of siderophore on CAS medium with Na-succinate as the C source. Of the three isolates, *K. planticola* and *P. vulgaris* showed the formation of yellow halo around the growth indicating their ability to produce siderophore. However, *B. subtilis* could not grow on CAS medium containing Na-succinate and

Plate 3. Siderophore production by PGPR isolates on chrome Azurol S medium. From left to right : *Bacillus subtilis* (scanty growth), *Klebsiella planticola* and *Proteus vulgaris*

Plate 4. Solubilization of tricalcium phosphate (TCP) in Pikovskaya medium. From left to right : *Proteus vulgaris* (significantly it solubilizes TCP from the centre of the colony), *Bacillus subtilis* and *Klebsiella planticola*

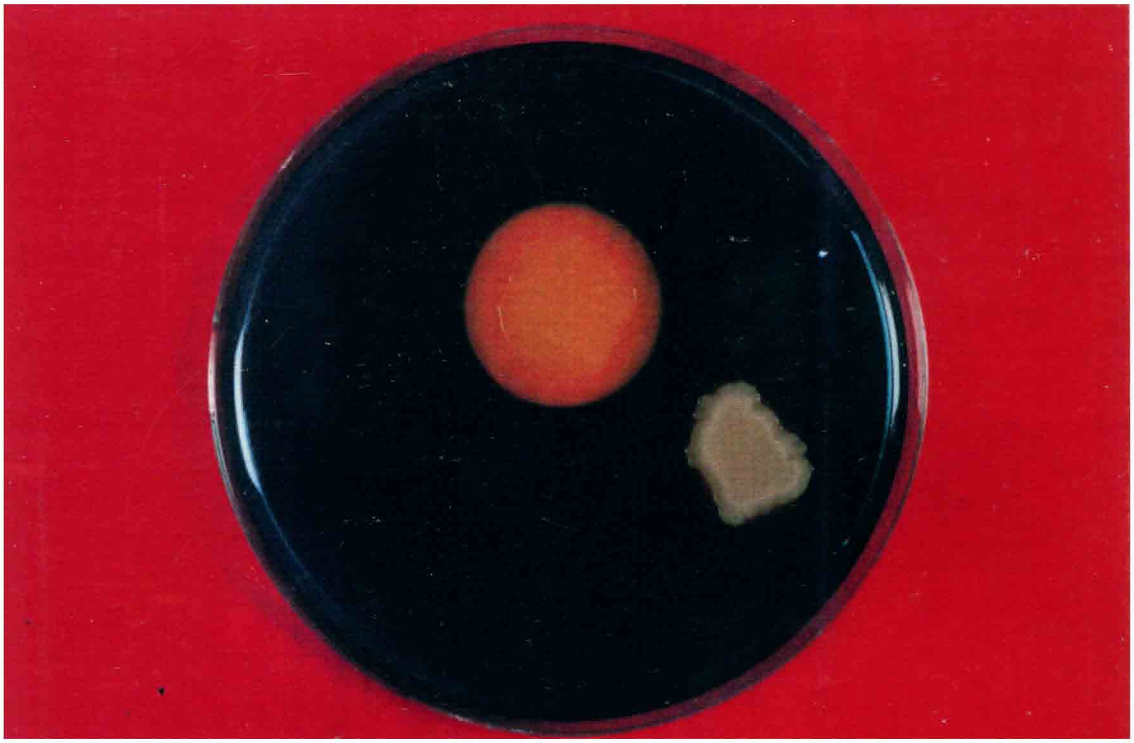


Table 4. Intrinsic antibiotic resistance profile of PGPR isolates

	PGR isolates	Antibiotic concentration (µg/ml)						
		Chloram-phenicol	Kana-mycin	Strepto-mycin	Ampi-cillin	Trimeth-oprin	Nalidixic acid	Tetra-cycline
1.	*B. subtilis*	200^r	200^r	200^r	200^r	200^r	200^r	100^r
2.	*K. planticola*	50^r	150^r	200^r	200^r	10^r	100^r	25^r
3.	*P. vulgaris*	50^r	150^r	200^r	200^r	25^r	150^r	100^r

numeral^r = Concentration at which isolate is resistant.

Table 5. IAA production (µg mg^{-1} protein) and per cent phosphate solubilization at different time intervals by PGPR isolates

Treatment	IAA	% P solubiliztion (days)			
		4	8	12	Average
1. *Bacillus subtilis*	ND	1.80	2.43	1.30	1.84
2. *Klebsiella planticola*	ND	4.00	4.80	1.96	3.50
3. *Proteus vulgaris*	253	2.78	3.32	1.38	2.50
4. Control	ND	0.70	1.03	0.26	0.66

ND = Not detected.

therefore it is difficult to comment on the ability or inability of the bacteria to produce siderophore (Plate 3).

4.2.5 Phosphate Solubilization

PGPR isolates were also tested for their ability to solubilize inorganic phasphate, Tricalcium phosphate, (TCP) both in liquid and solid Pikovskaya's medium. On plates, all the three isolates showed the formation of clearing zones around the growth. Maximum zone of clearance was produced by *B. subtilis* followed by *Klebsiella* and *Proteus* isolates (Plate 4).

Quantification of phosphate solubilization in liquid medium indicated that *Klebsiella planticola* was most efficient in TCP solubilization followed by *Proteus* and *Bacillus* isolates. Quantification of TCP solubilization was done at three stages after 4 and 8 and 12 days of incubation. The results revealed that highest solubilization was carried out within 8 days of incubation. However, on increasing the incubation time to 12 days, there was a decline in the amount of TCP solubilized (Table 5).

4.2.6 In vitro Antifungal Activity of PGPR Isolates

The isolates were screened for *in vitro* antifungal activities using 5 different fungi (Table 6) and potato dextrose agar medium. Isolates *B. subtilis* and *P. vulgaris* could inhibit all the five fungi tested whereas *K. planticola* could inhibit only four and could not inhibit *Colletotrichum falcatum* (Table 6 and Plate 5).

4.2.7 Starch Hydrolysis

Of the three PGPR isolates *B. subtilis* and *K. planticola* were able to hydrolyse starch as indicated by the appearance of colour on addition of Lugol's iodine *Proteus vulgaris* could not hydrolyse starch.

4.2.8 Citrate Utilization

All the three isolates could utilize citrate as indicated by the change of colour from green to blue of Simons citrate medium (Plate 6).

4.2.9 Nitrate Reduction

All the three were capable of reducing nitrate to nitrite (Table 7).

4.2.10 Ammonia Production

All the isolates were positive for ammonification.

Table 6. Antifungal reaction of PGPR isolates on PDA plates

	Fungi	Bacteria		
		B. subtilis	*K. planticola*	*P. vulgaris*
1.	*Macrophomina phaseolina*	+++	+++	+++
2.	*Fusarium solani*	++	+++	++
3.	*Fusarium moniliforme*	+++	+++	+++
4.	*Rhizoctonia solani*	+	++	+++
5.	*Colletotrichum falcatum*	+++	–	++

+++ = Very good growth
++ = Good growth
+ = Fair growth
– = No growth

Plate 5. Top left : Inhibition of *Fusarium solani* by *Bacillus subtilis*

Top right : Inhibition of *Colletotrichum falcatum* by *Bacillus subtilis*

Bottom left : Inhibition of *Fusarium moniliforme* by *Klebsiella planticola*

Bottom right : Inhibition of *Macrophomina phaseolina* by *Klebsiella planticola*

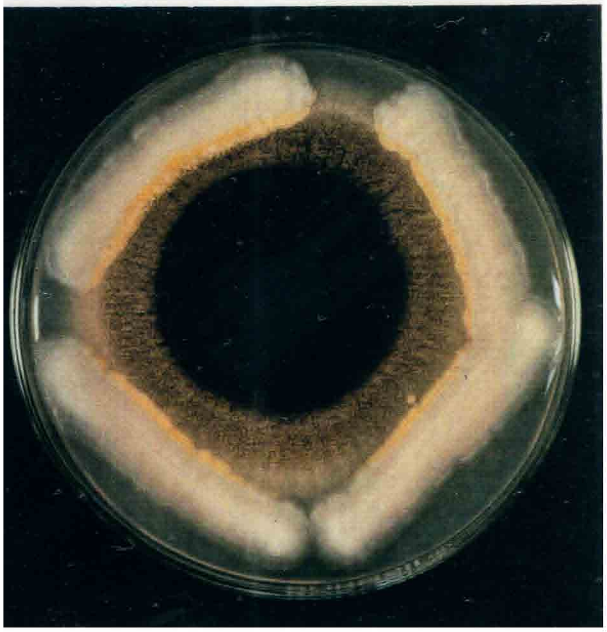


Plate 6. **Citrate utilization by three PGPR isolates. From left to right : Control, *Bacillus subtilis*, *Klebsiella planticola* and *Proteus vulgaris***

Table 7. Biochemical characterization of PGPR isolates

Organism	Citrate utilization	NO_3^- reduction	Production of			
			H_2S	NH_3	IAA	Siderophore
1. *Bacillus subtilis*	++	++	++	++	+	NG
2. *Klebsiella planticola*	++	++	++	++	+	++
3. *Proteus vulgaris*	++	++	++	++	++	++

++ = positive reaction
+ = moderately positive reaction
NG = No bacterial growth on medium

4.2.11 H_2S Production

All the three isolates showed production of H_2S gas.

4.2.12 In vitro Nitrogenase Activity

None of the cultures showed nitrogenase activity both in solid and liquid combined carbon medium.

4.3 Carrier based inoculant and shelf life

In order to test the efficacy of charcoal : soil mixture as a carrier for these PGPR isolates, survival of the three strains was tested for a period of 6 months at room temperature. The broth culture contained Ca 10^9 cells ml^{-1}. It maintained the similar population when mixed with carrier (10^9 cells g^{-1} carrier). All the three cultures followed the same pattern of survival in the carrier. There was a gradual decline in the counts of all the three bacteria till six months. Correspondingly there was a decline in the moisture content (%) from 40% at 0 time to 15.8% at the end of 6 month incubation (Fig. 1,2,3).

4.4 Response of Cereal Crops to Inoculation of PGPR

4.4.1 Wheat

A field trial was conducted using three N levels as main plots and five PGPR cultures along with one uninoculated control as sub plots. Of the five cultures, three were new PGPR isolates (*K. planticola, B. subtilis* and *P. vulgaris*) and two were already recommended biofertilizers (Azospirillum CDJA, *Azotobacter* CP15) for wheat. The efficiency of the new isolates were compared with the recommended strains. The plants were harvested after 45 days of plant growth and at maturity.

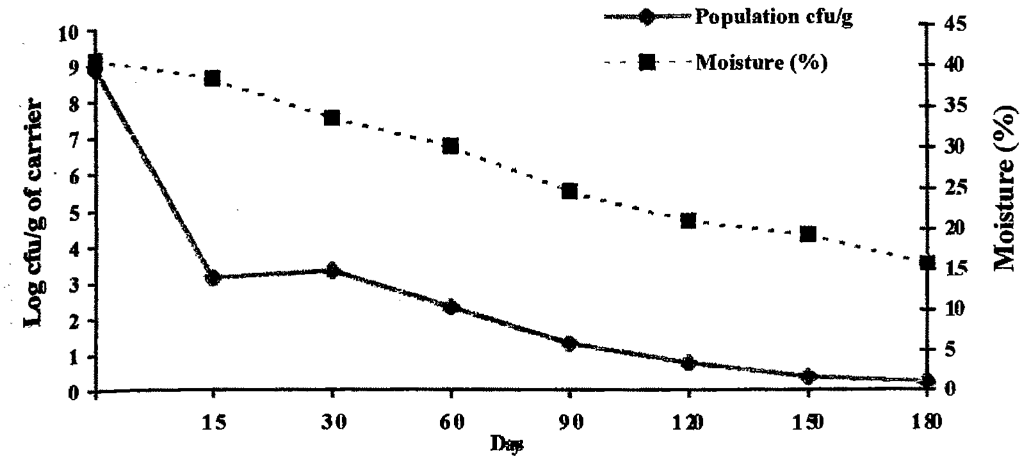


Fig.1 Survival of *Bacillus subtilis* as influenced by time and moisture content

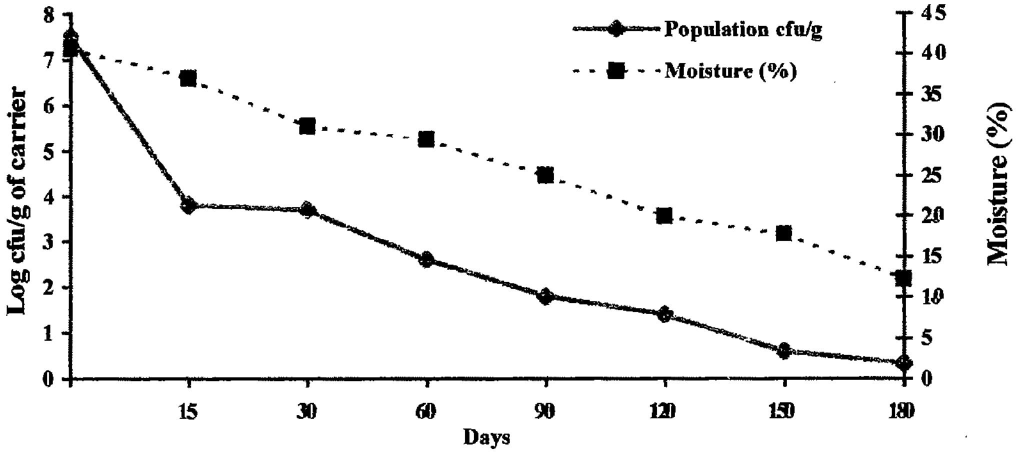


Fig. 2 Survival of *Klebsiella planticola* as influenced by time and moisture content

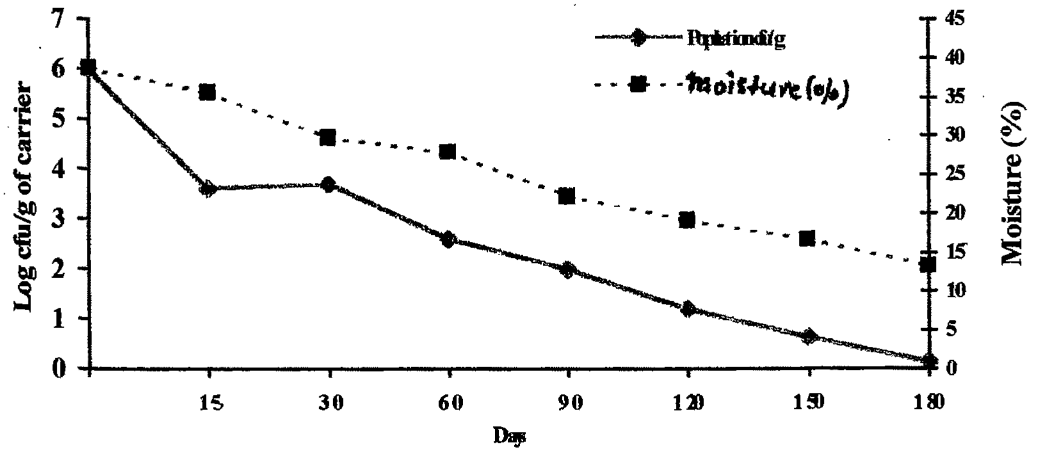


Fig.3 Survival of *Proteus vulgaris* as influenced by time and moisture content

Root and shoot dry weight was recorded after 45 days of plant growth (Table 8). Significant differences were observed between N levels and cultures whereas the N level X culture interactions were non significant. In general, the inoculation effects were more pronounced in plots not receiving any N fertilizer (N_0) At N_0 level, maximum root dry weight was obtained due to inoculation of *Azotobacter chroococcum* followed by *Azospirillum* CDJA. Among the new isolates, only *P. vulgaris* could significantly influence the root dry weight. At higher levels of nitrogen (N_{40} and N_{80}) there was no increase in root dry weight due to inoculation when compared to N_0 level. However, *Azotobacter* inoculation gave significant increase in root dry weight at both the levels when compared to uninoculated control.

The shoot dry weight after 45 days of plant growth did not revealed any significant differences both among the cultures and nitrogen levels. At N_0 level, *Azotobacter* inoculation gave the maximum shoot dry weight but statistically at par with uninoculated control (Table 8). Increase in nitrogen level (N_{40} and N_{80}) did not result in any significant increase in accumulation of shoot biomass for all treatments.

The population of inoculant bacteria were estimated in the rhizosphere of wheat after 45 days of plant growth. The results revealed that, in general, with the increase in the nitrogen level from N_0 to N_{80} there was an increase in the population of inoculant bacteria. At nitrogen level of N_0, *B. subtilis* could build up maximum population in the rhizosphere (Table 8). However, at a higher level of N_{40} maximum population was of *Azotobacter* whereas at N_{80} *Azospirillum* could colonize better than other bacteria.

Table 8. Inoculation effect of PGPR on root and shoot biomass of wheat cv. HD 2285 and their rhizospheric population at 45 days of plant growth (average of 4 replications)

Nitrogen level kg/ha	Treatment	Population cfu (x 10^5) g^{-1} soil	Root dry weight (g)	Shoot dry weight (g)
0	Uninoculated control	2.17	2.26	19.83
	Bacillus subtilis	25.05	2.55	19.42
	Klebsiella planticola	16.95	2.64	15.95
	Proteus vulgaris	13.73	2.79	18.62
	Azospirillum brasilinse (CDJA)	16.83	2.91	21.58
	Azotobacter chroococcum (CBD15)	20.33	3.33	22.29
40	Uninoculated control	2.53	1.84	19.17
	Bacillus subtilis	20.00	1.94	20.07
	Klebsiella planticola	22.63	2.37	20.10
	Proteus vulgaris	14.45	1.83	19.80
	Azospirillum brasilinse (CDJA)	23.70	1.87	18.48
	Azotobacter chroococcum (CBD15)	26.23	2.70	18.77
80	Uninoculated control	2.63	2.31	18.63
	Bacillus subtilis	28.25	1.61	16.30
	Klebsiella planticola	21.65	2.25	17.75
	Proteus vulgaris	17.35	2.26	18.32
	Azospirillum brasilinse (CDJA)	34.67	2.10	18.74
	Azotobacter chroococcum (CBD15)	28.23	3.05	20.91
	C.V. %	34.0	22.8	12.7
	$CD_{0.05}$			
	(a) Nitrogen level (N)	3.89	0.58	NS
	(b) Inoculant (I)	4.96	0.44	NS
	(c) N x I	NS	NS	NS

At maturity, the plant height of the standing crop was measured and grain and straw yield was recorded. The plant height did not exhibit any significant variation due to inoculation or fertilization (Table 9).

The straw yield was influenced significantly by application of nitrogen fertilizer. However, variation was not observed between N_{40} and N_{80} level. Among the cultures, *K. planticola* inoculation at N_0 level resulted in maximum increase of 16% in straw yield, whereas N_{40} and N_{80} level, maximum increase was obtained due to inoculation of *Azospirillum* CDJA and *Azotobacter* respectively (Table 9).

Grain yield was significantly increased with N fertilizer application. Rhizobacteria inoculated plants increased wheat grain yield at about 2 to 5.4%. But when they were supplemented with 40 kg N ha^{-1} the yield could be increased by over 25 per cent. Of all the treatments, maximum grain was obtained in plots inoculated with *A. brasilense* CDJA and amended with 40 kg N ha^{-1}. It was closely followed by treatment inoculated with *Proteus vulgaris* at the same nitrogen level i.e. N_{40} (Table 9). The results clearly revealed that the new PGPR isolates were not superior than the recommended biofertilizer strains for inoculation of wheat crop.

4.4.2 Teff (Eragrostis tef)

Teff crop was grown in earthen pots and two levels of nitrogen 0 and 46 kg N/ha were maintained. PGPR inoculation was done both in the absence and presence of nitrogen fertilizer. The data was recorded on plant height, head size, dry matter and seed yield and presented in Table 10.

Table 9. The effect of PGPR on plant height, straw and grain yield of wheat cv. HD 2285 (average of 4 replications

Nitrogen level kg/ha	Treatment	Plant height (cm)	Mean yield (kg/ha)		% increase over control Grain yield
			Straw	Grain	
0	Uninoculated control	83.0	8600	3911	—
	Bacillus subtilis	85.3	9662	4060	3.8
	Klebsiella planticola	85.4	9987	4123	5.4
	Proteus vulgaris	84.6	8275	3645	-6.8
	Azospirillum brasilinse (CDJA)	84.8	9087	4032	3.1
	Azotobacter chroococcum (CBD15)	84.2	9150	3983	1.9
40	Uninoculated control	91.8	10862	4476	—
	Bacillus subtilis	88.0	11175	4576	2.2
	Klebsiella planticola	84.4	10428	4287	-4.2
	Proteus vulgaris	88.7	11925	4842	8.0
	Azospirillum brasilinse (CDJA)	91.7	12175	4913	9.8
	Azotobacter chroococcum (CBD15)	88.0	10600	4437	-0.86
80	Uninoculated control	89.0	10575	4026	—
	Bacillus subtilis	87.8	11025	4340	7.8
	Klebsiella planticola	90.1	11450	4353	8.1
	Proteus vulgaris	86.6	11000	4397	9.2
	Azospirillum brasilinse (CDJA)	88.6	10700	4535	12.6
	Azotobacter chroococcum (CBD15)	89.1	11625	4457	10.7
	C.V. %	5.10	13	13	
	$CD_{0.05}$				
	(a) Nitrogen level (N)	2.73	1060	422	
	(b) Inoculant (I)	NS	NS	NS	
	(c) N x I	NS	NS	NS	

Table 10. Inoculation effect of PGPR isolates on plant height, head size, biomass and grain yield of teff cv. DZ-01-354 (average of 3 replications)

Nitrogen level kg/ha	Treatment	Plant height (cm)	Head (cm)	Mean yield (g/pot)		% increase over control grain yield
				Biomass	Grain	
0	Uninoculated control	83.6	25.3	56.9	1.19	—
	Bacillus subtilis	94.6	26.0	80.8	2.18	83.2
	Klebsiella planticola	91.5	27.9	68.8	1.56	31.1
	Proteus vulgaris	86.4	23.5	61.6	0.89	-25.2
	Azospirillum brasilinse	94.0	27.3	51.3	0.87	-26.9
	Azotobacter chroococcum	89.4	28.5	64.7	1.22	2.5
46	Uninoculated control	89.4	27.7	47.4	0.65	—
	Bacillus subtilis	90.8	29.2	47.4	0.65	0.0
	Klebsiella planticola	101.5	29.1	50.0	0.65	0.0
	Proteus vulgaris	89.0	31.2	57.5	1.34	106.2
	Azospirillum brasilinse	100.0	30.3	59.5	1.02	56.9
	Azotobacter chroococcum	89.6	23.5	50.1	1.11	70.8
	C.V. %	13.7	27.0	23.4	21.1	
	$CD_{0.05}$					
	(a) Nitrogen level (N)	NS	NS	NS	0.38	
	(b) Inoculant (I)	NS	NS	NS	NS	
	(c) N x I	NS	NS	NS	NS	

Plant height showed no variations both among the nitrogen levels and PGPR isolates. In the absence of nitrogen amendment, maximum plant height was obtained in treatment inoculated with *B. subtilis* closely followed by *Azospirillum* treatment. However, in the presence of nitrogen, *K. planticola* followed by *Azospirillum* influenced the plant height of teff plant.

Head size was influenced due to application of 46 kg N/ha. The uninoculated but N fertilized control showed an increase of 9.5% over uninoculated and unfertilized control. In the absence of N fertilizer, inoculation of *Azotobacter* recorded the maximum head size and was 12.7% more than that obtained in uninoculated control. However, in the presence of 46 kg N/ha, maximum head size was obtained in treatment inoculated with *Proteus vulgaris*.

The biomass and seed yield was not influenced by application of nitrogen fertilizer. In general, for all treatments, lower values were obtained for both the parameters in the presence of nitrogen fertilizer. In the absence of nitrogen, inoculation of *B. subtilis* resulted in maximum dry matter and seed yield and was significantly higher than all other treatments. In the presence of nitrogen fertilizer, inoculation of *Proteus vulgaris* resulted in maximum seed yield and was 106 per cent higher than the uninoculated control.

4.5 Response of Leguminous Crops to Coinoculation of Rhizobium and PGPR

The three PGPR isolates *K. planticola*, *B. subtilis* and *P. vulgaris* were tested individually and in combination with specific rhizobial strains on four different pulse crops namely soybean, chickpea, mungbean and lentil. The main aim of these field experiments was to identify whether these three PGPR isolates can also be defined as nodulation promoting rhizobacteria (NPR).

4.5.1 Soybean

The effect of PGPR strains inoculated singly and in combination with *Bradyrhizobium* was studied in a field experiment using soybean cultivar Pusa 22 (Plates 7 and 8). The results are presented in Table 11, 12. Nodule number and biomass was significantly influenced due to inoculation of PGPR isolates both singly and in combination with *Bradyrhizobium* strain SB271. Maximum nodule number and mass was obtained due to single inoculation of *B. subtilis* followed by *Bradyrhizobium* alone (Table 11). Among the coinoculation treatments combination of *Bradyrhizobium* and *P. vulgaris* produced the maximum nodule number and nodule mass. Inoculated treatments did not differ significantly from the uninoculated control with respect to root and shoot dry weight. Among the single inoculated treatments, *Klebsiella planticola* gave the maximum root and shoot dry biomass. However, in coinoculation with *Bradyrhizobium*, *P. vulgaris* performed the best. Maximum grain yield was obtained in plots inoculated with a combination of *Bradyrhizobium* SB271 and *B. subtilis*. However, the increase was only 19 per cent over the uninoculated control. The total N content was influenced significantly due to all inoculations either single or in combination. Maximum N was accumulated in plants coinoculated with *Bradyrhizobium* and *B. subtilis* (Table 12).

4.5.2 Lentil

Maximum nodule numbers and mass was obtained in plots

Plate 7. Field view of soybean crop. From top to bottom : Uninoculated plot, *Rhizobium* inoculated and *Bacillus subtilis* inoculated


CONTROL
(UNINOCULATED)


B.
RHIZOBIUM
(STRAIN)


BACILLUS
SUBTILIS

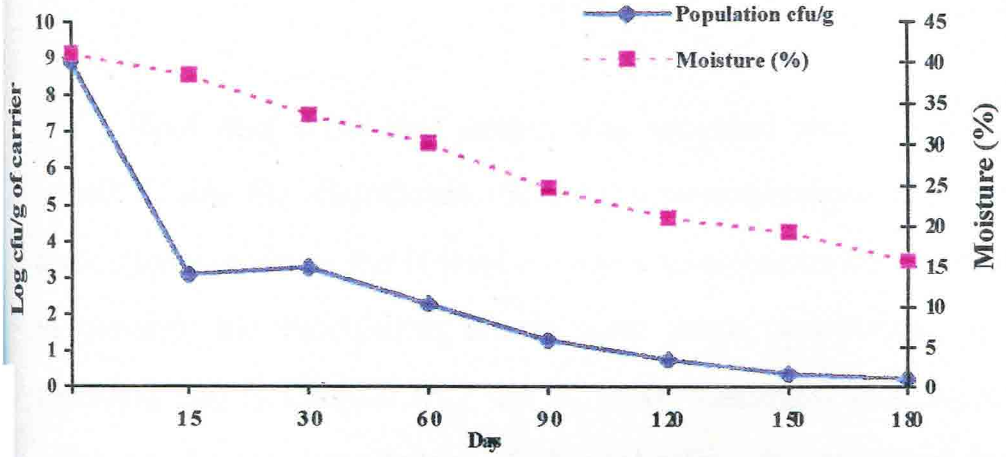


Fig.1 Survival of *Bacillus subtilis* as influenced by time and moisture content

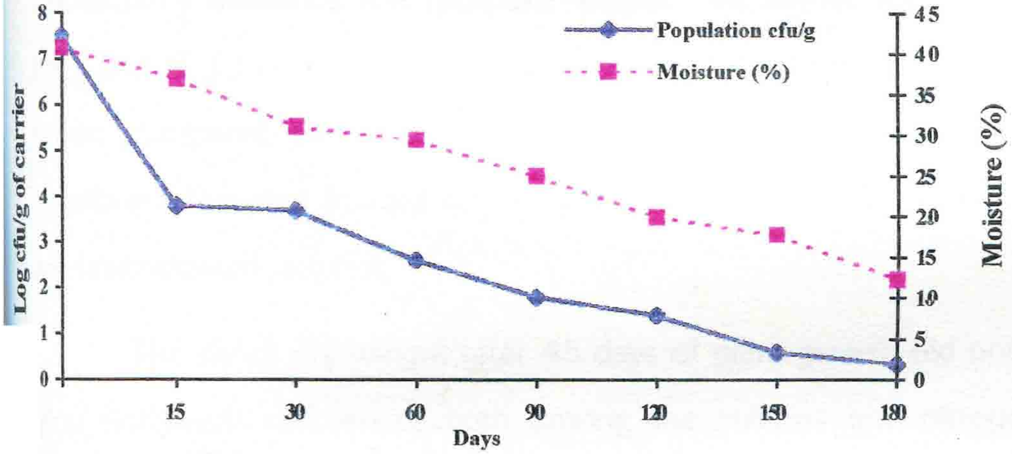


Fig. 2 Survival of *Klebsiella planticola* as influenced by time and moisture content

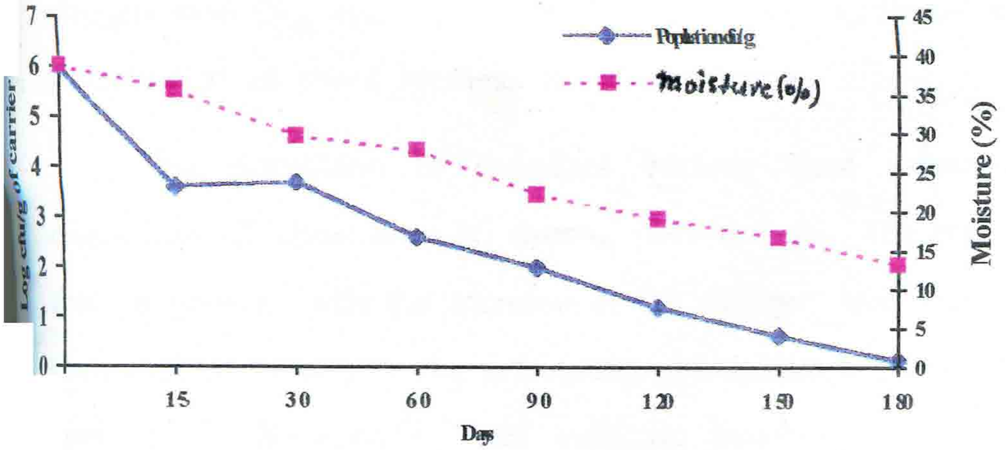


Fig.3 Survival of *Proteus vulgaris* as influenced by time and moisture content

Plate 8. Field view of soybean crop

Top : *Klebsiella planticola* inoculated

Bottom : *Proteus vulgaris* inoculated


KLEBSIELLA
PLANTICOLA


PROTEUS
VULGARIS

Table 11. Coinoculation effect of PGPR isolates and *Bradyrhizobium japonicum* (SB 271) on nodulation and growth of soybean cv. (average of 3 replications)

Treatment	Nodule number	Nodule weight (g)	Root dry weight (g)	Shoot dry weight (g)
1. Uninoculated control	0	0.00	4.70	10.55
2. *Bacillus subtilis* (Bs)	99	0.85	5.61	12.53
3. *Kelbsiella planticola* (Kp)	0	0.02	5.73	15.35
4. Proteus vulgaris (Pv)	0	0.00	4.62	10.47
5. *Bradyrhizobium japonicum* (SB 271)	93	0.64	4.63	11.83
6. SB 271 x Bs	64	0.47	4.70	10.48
7. SB 271 x Kp	56	0.40	4.34	10.30
8. SB 271 x Pv	79	0.81	6.81	14.70
9. 80 kg N/ha	0	0.00	5.68	13.65
CV %	32	35.9	26.7	19.6
$CD_{0.05}$	40.64	0.34	NS	NS

Table 12. Coinoculation effect of PGPR isolates and *Bradyrhizobium japonicum* (SB 271) on plant height N-content and grain yield of soybean cv. (average of 3 replications)

Treatment	Plant height (cm)	N-plant mg/plant	Grain yield kg/ha	% increase over control
1. Uninoculated control	90.3	10.94	1705	—
2. *Bacillus subtilis* (Bs)	94.8	21.05	1842	8.1
3. *Kelbsiella planticola* (Kp)	89.8	20.77	1798	5.5
4. Proteus vulgaris (Pv)	87.1	21.86	1648	-3.3
5. *Bradyrhizobium japonicum* (SB 271)	90.7	22.11	1886	10.7
6. SB 271 x Bs	84.7	35.22	2032	19.2
7. SB 271 x Kp	93.3	28.68	1878	10.2
8. SB 271 x Pv	38.1	25.96	1789	5.0
9. 80 kg N/ha	96.0	12.50	1648	-3.3
CV %	8.9	18.2	15	
$CD_{0.05}$	NS	4.66	NS	

inouclations of *Bacillus* and *Rhizobium* treatment. However, the nitrogenase activity was maximum for treatment where Rhizobium was inoculated alone (Table 13). The results of field experiment with lentil crop are presented in Table 13 and 14. The root and shoot dry weight of lentil plants showed no variations as a result of bacterial inoculation. The highest values of root and shoot dry weight were obtained due to coinoculation of *Rhizobium* and *Proteus vulgaris* (Table 13). None of the PGPR isolates either singly or in coinoculation with *Rhizobium* strain could significantly influence the growth and yield of lentil crop. However, inoculation of *B. subtilis* increased lentil seed yield by 6.8% (Table 14).

4.5.3 Mungbean

The results of field experiment using mungbean as a test crop are presented in Table 15 and 16. The plants were harvested after 40 days of plant growth and nodulation data was recorded. The results showed a significant increase in the number of nodules due to inoculation of *Rhizobium* strain M11, single inoculations of PGPR isolates except *K. planticola* and dual inoculation of all PGPR isolates with *Rhizobium* M11. Maximum number of nodules were obtained due to dual inoculation of M11 and *Bacillus subtilis* (Table 15).

Shoot dry weight and grain yield were also recorded. All the treatments were statistically at par with respect to both the parameters. Maximum shoot biomass was obtained in treatment inoculated with a combination of *B. subtilis* and *Rhizobium* M11 whereas maximum grain yield was recorded in treatment inoculated with *Bacillus subtilis* alone (Table 15 and 16).

Table 13. Coinoculation effect of PGPR isolates and *Rhizobium* (L-12-87) on growth, nodulation and nitrogenase activity of lentil (average of 3 replications)

Treatment	Nodule number	Nodule weight (g)	Root dry weight (g)	Shoot dry weight (g)	ARA μ moles C_2H_4 hr^{-1} g^{-1} nodule
1. Uninoculated control	62	0.32	0.17	2.41	161.7
2. *Bacillus subtilis* (Bs)	89	0.37	0.14	2.51	186.6
3. *Kelbsiella planticola* (Kp)	57	0.20	0.15	2.64	192.9
4. Proteus vulgaris (Pv)	81	0.32	0.15	2.08	203.1
5. *Rhizobium* (L-12-87) (R)	91	0.31	0.15	2.57	238.8
6. R x Bs	102	0.44	0.19	2.53	131.9
7. R x Kp	75	0.24	0.14	2.07	205.6
8. R x Pv	104	0.46	0.20	2.70	158.8
CV %	16	10.88	19.2	19.6	35.6
$CD_{0.05}$	NS	NS	NS	NS	NS

Table 14. Coinoculation effect of PGPR isolates and *Rhizobium* (L-12-87) on biomass, grain yield and percentage increase of grain yield of lentil (average of 3 replications)

Treatment		Yield kg/ha		% increase in grain yield
		Biomass	Grain	
1.	Uninoculated control	4992	1970	—
2.	*Bacillus subtilis* (Bs)	5303	2103	6.8
3.	*Kelbsiella planticola* (Kp)	5096	2014	2.3
4.	Proteus vulgaris (Pv)	4977	1955	-0.8
5.	*Rhizobium* (L-12-87) (R)	5259	2029	3.0
6.	R x Bs	4296	1733	-12.0
7.	R x Kp	4948	2000	1.5
8.	R x Pv	5244	2014	2.3
	CV %	9	8	
	$CD_{0.05}$	NS	NS	

Table 15. Coinoculation effect of PGPR isolates and mungbean *Rhizobium* (M-11) on nodulation and growth of mungbean cv. Pusa 9027 (average of 3 replications)

	Treatment	Nodule number	Shoot dry weight (g)
1.	Uninoculated control	137	4.90
2.	*Bacillus subtilis* (Bs)	219	4.50
3.	*Kelbsiella planticola* (Kp)	98	5.13
4.	Proteus vulgaris (Pv)	122	4.47
5.	*Rhizobium* (M-11)	275	5.30
6.	M-11 x Bs	372	5.13
7.	M-11 x Kp	238	5.03
8.	M-11 x Pv	160	4.87
	CV %	27	15.5
	$CD_{0.05}$	NS	NS

Table 16. Coinoculation effect of PGPR isolates and mungbean *Rhizobium* (M-11) on grain yield and percentage increase of grain yield of mungbean (average of 3 replications)

Treatment		Grain yield kg/ha	% increase over control
1.	Uninoculated control	920	—
2.	*Bacillus subtilis* (Bs)	1069	16.2
3.	*Kelbsiella planticola* (Kp)	930	1.1
4.	Proteus vulgaris (Pv)	972	5.7
5.	*Rhizobium* (M-11)	951	3.4
6.	M-11 x Bs	965	4.9
7.	M-11 x Kp	857	-6.8
8.	M-11 x Pv	972	5.7
	CV %	9	
	$CD_{0.05}$	NS	

4.5.4 Chickpea

A field experiment was conducted using an efficient *Rhizobium* strain (F75) specific to chickpea along with three PGPR isolates. The plant samples were harvested after 45 days of plant growth and observation were made on root and shoot dry biomass, nodule number and nitrogenase activity.

The root dry weight was not influenced significantly due to single and coinoculation treatments. Combination of *B. subtilis* and *Rhizobium* F75 gave the maximum root biomass (Table 17).

The shoot dry weight was not influenced significantly due to all the treatments except for single inoculation of *Bacillus subtilis*. A combination of *B. subtilis* and *Rhizobium* depressed the accumulation of shoot biomass (Table 17).

The nodule number was significantly influenced due to coinoculation of *Rhizobium* and *Bacillus subtilis*. All other treatments were statistically at par with the uninoculated control (Table 17).

The root nodule nitrogenase activity showed variations due to inoculation treatments (Table 17). Except for single inoculation of *B. subtilis,* all other treatments gave very high values of nitrogenase activity as compared to uninoculated control. Coinoculation of *Rhizobium* with three PGPR isolates was superior although statistically insignificant when compared to single inoculation of PGPR isolates and *Rhizobium* strain (Table 17).

Table 17. Coinoculation effect of PGPR isolates and *Mesorhizobium ciceri* F75 on growth, nodulation and nitrogenase activity of chickpea cv. Pusa 256 (average of 3 replications)

Treatment	Nodule number	Nodule weight (g)	Root dry weight (g)	Shoot dry weight (g)	ARA µ moles C_2H_4 hr^{-1} g^{-1} nodule
1. Uninoculated control	88	3.77	1.6	19.4	19.1
2. *Bacillus subtilis* (Bs)	88	3.44	1.6	23.1	6.5
3. *Kelbsiella planticola* (Kp)	92	2.67	1.6	21.6	108.0
4. Proteus vulgaris (Pv)	74	2.87	1.4	20.2	99.8
5. *Rhizobium* (F 75)	74	3.15	1.4	19.4	87.4
6. F 75 x Bs	141	5.50	2.2	18.9	114.9
7. F 75 x Kp	98	2.75	1.4	19.5	120.5
8. F 75 x Pv	70	3.15	1.5	18.5	108.5
CV %	33	31.5	23.1	18.9	34.3
$CD_{0.05}$	NS	NS	NS	NS	NS

The grain yield was recorded at maturity and the yield increase due to inoculation varied from 1.2 to 14.3 per cent (Table 18). The yield increases were statistically non significant among the treatments and maximum increase of 14.3 per cent was obtained due to single inoculation of *Rhizobium* strain F 75.

4.6 Response of Oil Seed Crops to PGPR Inoculation

Green house experiments were conducted to assess the influence of new PGPR isolates on the growth and yield of two oil seed crops, that is sunflower and mustard. In addition to the three PGPR isolates, already in use biofertilizers like *Azotobacter* and *Bacillus polymyxa* for mustard and *Azospirillum* for sunflower were also included as a reference for comparison.

4.6.1 Sunflower

A pot experiment was conducted to evaluate three PGPR isolates along with a strain of *Azospirillum* CDJA on growth and yield of sunflower. The data was recorded on plant height, shoot dry weight, head diameter and seed yield. Large variations were observed among the replicates and the results were inconclusive. For all the parameters, there were no significant differences among the treatments (Table 19). However, the maximum head diameter and seed yield was obtained in treatments inoculated with *K. planticola* and *B. subtilis* respectively (Table 19).

4.6.2 Mustard

A pot experiment was conducted for two consecutive years to assess the influence of PGPR strains on growth and yield of mustard. The average

Table 18. Coinoculation effect of PGPR isolates and Meso*rhizobium ciceri* F 75 on grain yield of chickpea cv. Pusa 256 (average of 3 replications)

	Treatment	Grain yield kg/ha	% increase over control
1.	Uninoculated control	2170	—
2.	*Bacillus subtilis* (Bs)	2325	7.2
3.	*Kelbsiella planticola* (Kp)	2196	1.2
4.	Proteus vulgaris (Pv)	2416	11.3
5.	*Rhizobium* (F 75)	2480	14.3
6.	F 75 x Bs	2144	-1.2
7.	F 75 x Kp	2313	6.6
8.	F 75 x Pv	2248	3.6
	CV %	15	
	$CD_{0.05}$	NS	

Table 19. Inoculation effect of PGPR isolates on shoot dry matter yield, plant height, head diameter and grain yield of sunflower cv. Mordan (Average of 3 replications)

Treatment	Shoot dry weight (g)	Plant height (cm)	Head diameter (cm)	Grain yield (g/plant)	% increase over control grain yield
40 kg N/ha	23.9	67.0	7.2	5.8	—
40 kg N/ha + *B. subtilis*	32.8	70.0	7.8	6.3	8.6
40 kg N/ha + *K. planticola*	32.4	75.3	9.5	5.3	
40 kg N/ha + *P. vulgaris*	26.5	63.3	7.3	4.9	
40 kg N/ha + *A. brasilense*	26.1	69.8	6.6	3.8	
80 kg N/ha	24.2	66.8	6.9	3.9	
CV %	18.0	13.8	3.7	15.5	
$CD_{0.05}$	NS	NS	NS	NS	

of two year data is presented in Table 20. Like sunflower, in case of mustard crop also the differences among treatments were statistically not significant. Both in the presence and absence of nitrogen fertilizer, inoculation of *Azotobacter* gave maximum values of root and shoot dry weight. Differences were observed in the dry biomass among plants growing with (30 kg N ha^{-1}) and without nitrogen, although statistically they were insignificant. *Bacillus subtilis* could establish well in the rhizosphere of mustard in the absence of nitrogen. However, in the presence of nitrogen, *Klebsiella planticola* recorded the maximum population could closely followed by *Bacillus subtilis* (Table 20). Biomass kand grain yield of mustard showed non-significant variations both among the inoculated treatments and nitrogen levels. At both the levels of N *Proteus vulgaris* resulted in maximum per cent increase in grain yield (Table 21).

Table 20. Inoculation effect of PGPR on root and shoot biomass of mustard cv. Pusa Bold and their rhizospheric population at 45 days of plant growth (average of 4 replications)

Nitrogen level kg/ha	Treatment	Population cfu (x 10^5) g^{-1} soil	Root dry weight (g)	Shoot dry weight (g)
0	Uninoculated control	1.8	1.53	6.05
	Bacillus subtilis	62.5	2.45	6.28
	Klebsiella planticola	17.2	1.59	5.49
	Proteus vulgaris	14.7	1.21	4.70
	Bacillus polymyxa	20.1	1.62	5.80
	Azotobacter chroococcum	21.0	1.89	6.75
30	Uninoculated control	2.0	1.72	6.89
	Bacillus subtilis	26.6	1.65	6.99
	Klebsiella planticola	27.2	1.48	7.44
	Proteus vulgaris	15.1	1.81	7.17
	Bacillus polymyxa	12.6	1.77	7.27
	Azotobacter chroococcum	37.0	2.18	8.42
	C.V. %	36.5	27.4	32.5
	$CD_{0.05}$			
	(a) Nitrogen level (N)	8.3	NS	NS
	(b) Inoculant (I)	14.4	NS	NS
	(c) N x I	NS	NS	NS

Table 21. Inoculation effect of PGPR isolates on plant height, biomass and grain yield of mustard cv. Pusa Bold (average of 3 replications)

Nitrogen level kg/ha	Treatment	Plant height (cm)	Mean yield (g/pot)		% increase over control Grain yield
			Biomass	Grain	
0	Uninoculated control	61.16	7.00	0.81	—
	Bacillus subtilis	66.42	8.47	1.14	40.7
	Klebsiella planticola	68.45	7.25	1.11	37.0
	Proteus vulgaris	65.00	9.05	1.15	42.0
	Bacillus polymyxa	16.62	7.72	0.75	-7.4
	Azotobacter chroococcum	65.60	6.52	0.75	-7.4
30	Uninoculated control	64.61	7.00	0.82	—
	Bacillus subtilis	60.48	6.62	0.75	-8.5
	Klebsiella planticola	72.20	8.08	1.05	28.1
	Proteus vulgaris	71.38	8.52	1.06	29.3
	Bacillus polymyxa	68.50	7.81	0.72	-12.2
	Azotobacter chroococcum	69.73	7.58	0.75	-8.5
	C.V. %	10.1	23.4	17.3	
	$CD_{0.05}$				
	(a) Nitrogen level (N)	NS	NS	NS	
	(b) Inoculant (I)	NS	NS	NS	
	(c) N x I	NS	NS	NS	

5.0 DISCUSSION

5.1 Biochemical Reactions

Three PGRR isolates namely *Proteus vulgaris, Klebsiella planticola* and *Bacillus subtilis* were isolated from rhizosphere of sunflower. These isolates were tested both in pots and fields on crops like sunflower, maize and cotton and significant increase in yields were reported (Malik *et al.,* 1996). However, the isolates were not characterized biochemically and the plant growth promoting attributes were not studied. Therefore, in the present investigation attempt was made to characterize the isolates biochemically and also to look for their effect on cereal, oilseed and leguminous crops.

All the three isolates were found positive for citrate utilization, nitrate reduction, ammonia and H_2S production. These characters are typical of the genera *Proteus, Klebsiella* and *Bacillus* (Buchanan and Gibbens, 1974). These rhizospheric isolates were found to grow on wide range of C sources. Of the 16 sugars tested, *Bacillus subtilis* was found to grow on all but not on mannitol. *Klebsiella* could grow on all except lactose and maleic acid @ 10 g^{-1} l and *Proteus vulgaris* could utilize all C sources except lactose. The ability of these bacteria to utilize large number of C source suggests their rhizospheric competence. Large population of *Pseudomonas* in the rhizosphere is also explained on the basis of their wide adaptability to utilize root exudates including simple and complex carbohydrates by the production of extracellular enzymes (Weller *et al.,* 1988; Hebbar *et al.,* 1992).

Intrinsic antibiotic resistance pattern showed variation among the three isolates. Intrinsic antibiotic resistant pattern can be used as a genetic marker for identification of strain and for ecological studies (Josey *et al.*, 1979). All the three PGPR isolates could be distinguished from each other through their resistance to varying concentrations of trimethoprin, nalidixic acid and tetracycline (Table 4). This property helped in preparing selective media for establishment studies on these bacteria in pots and field.

The PGPR isolates were further characterized for their plant growth promoting attributes like nitrogen fixation, IAA production, phosphate solubilization, siderophore production and antifungal activity.

Acetylene reduction assay (ARA) was carried out using combined carbon medium. The experiment was repeated three times giving different incubations ranging from 4 hrs to 24 hrs, but none of the cultures showed nitrogenase activity. Earlier reports on the same bacteria have shown that *P. vulgaris* and *K. planticola* to be nitrogen fixing bacteria (Malik *et al.*, 1995; Malik *et al.*, 1996). However, their conclusion was not based on nitrogenase assay but instead on estimation of total N through kjeldahl method (Malik *et al.*, 1995). *Klebsiella planticola* have been reported by many workers as a nitrogen fixing bacteria associated with leaves or leaf sheaths of various plants (Bagley *et al.*, 1981; Banaquio *et al.*, 1983; Ladha *et al.*, 1983).

The ability of the three rhizobacteria to solubilize tricalcium phosphate (TCP) under *in vitro* conditions was also tested. All the three PGPR isolates could solubilize TCP to varying levels. Maximum solubilization was observed by *K. planticola* after 8 days of incubation (Table 5). Of the three PGPR isolates, bacteria belonging to the genus *Bacillus* have been reported to solubilize TCP and other insoluble forms (Illmer and Schinner, 1995; Singh and Kapoor, 1998). However, reports on the ability of *K. planticola* and *P. vulgaris* to solubilize phosphorus are not available. This is one of the important attribute of rhizobacteria to be designated as plant growth promoting rhizobacteria.

Production of plant growth hormones particularly IAA has long been considered as an important attribute of PGPR strain. Of the three PGPR isolates tested, only *Proteus vulgaris* was found to produce IAA in small amounts only in the presence of its precursor, tryptophan (table 5). PGPR have been found to produce IAA in varying amounts (Barea *et al.,* 1976; Tien *et al.,* 1979; Prikryl *et al.,* 1985). There are many reports in the literature correlating the ability of strain to produce IAA and growth promotion of plants (Dubeikovsky *et al.,* 1993; Noel *et al.,* 1996). The amount of IAA produced by PGPR is also a critical factor. Loper and Schroth (1986) reported that bacterization of seeds with two isolates 7SR5 and 7SR13 producing high levels of IAA (5-10 mg ml^{-1}) resulted in reduced root elongation and increased shoot : root ratio of sugarbeet.

The three PGPR isolates were found to have antifungal activities and could inhibit five soil borne fungi tested (Table 6). Thus PGPR isolates can indirectly promote growth by suppressing the disease causing fungal pathogens. Several genera have shown their potential for biocontrol both under *in vivo* and *in vitro* conditions. Important among these are *Agrobacterium* (Kerr, 1972); *Enterobacter* (Roberts *et al.,* 1976); *Pseudomonas* (Gomes *et al.,* 1996; Pal *et al.,* 2000), *Bacillus* (Asaka and Shoda, 1996) and *Serratia* (Berg and Behl, 1997). Several mechanisms for disease suppression have been proposed including production of antifungal metabolites, siderophores, nutrient competition and niche exclusion (Chen *et al.*, 1995; Saxena *et al.*, 2000). Of the three isolates, *Proteus vulgaris* and *Klebsiella planticola* produced siderophores (Plate ...). However, siderophore production as a mechanism for fungal control could be ruled out for these bacteria as they could suppress fungal growth in an iron rich medium (PDA).

5.2 Carrier-Based Inoculant and Shelf Life

With an objective to develop a delivery system for these PGPR, already existing technology of preparing carrier based inoculants was tested. The carrier used was charcoal : soil (3 : 1) which has been in use at IARI for commercial production of inoculants. In this carrier, rhizobia and *Azotobacter* have been shown to survive in much greater numbers and even after 6 months more than the minimum standards of 10^7 cells g^{-1} of inoculant was maintained (Jauhri *et al.*, 1979). In the present study, all the three isolates could survive in the carrier at room temperature for a period of six months. However, their shelf life was only upto 3 months as there was a fall in population below the minimum standards of 10^7 cells g^{-1} of inoculant. Thus there is a need to develop an alternate carrier material which can extend the shelf life of these isolates to a minimum of six months.

5.3 Response of Crop Plants to PGPR Inoculation

Three new PGPR isolates, namely, *Klebsiella planticola, Proteus vulgaris* and *Bacillus subtilis* were chosen for the present study as they have been reported to increase yield of sunflower, wheat, cotton and sorghum (Malik *et al.*, 1996; Singh and Panwar, 1997). The major for various crop plants. Thus crop plants belonging to three major groups, cereals (wheat and teff), pulses (lentil, chickpea, mungbean and soybean) and oil seed crops (sunflower and mustard) were selected for the present investigation. Of these various crops, five were evaluated in field trials (wheat, lentil, chickpea, mungbean and soybean) and three in pot trials (teff, sunflower and mustard). The results obtained from these studies are discussed in this chapter.

5.3.1 *Cereal Crops*

5.3.1.1 *Wheat*

Wheat is one of the most important and high fertilizer demanding crops of the world. Several investigations have been carried out to reduce the application of costly nitrogen fertilizer through the use of plant growth promoting rhizobacteria particularly belonging to the genera *Azospirillum, Azotobacter, Serratia* and *Psudomonas* (Okon and Labandera-Gonzalez 1994; de Frietas and Germida 1990; Van Overbeek *et al.,* 1995; Saubidet and Barneix, 1998; Saxena and Tilak 1998). In the present investigation, after 45 days of plant growth, maximum root and shoot biomass was obtained due to inoculation of *Azotobacter* at all the levels of nitrogen (0, 40 and 80 kg N ha^{-1}). *Azotobacter* has been reported to influence wheat growth (Saxena and Tilak, 1998). Among the new PGPR isolates tested, *Proteus vulgaris* was found to influence the biomass accumulation to a limited extent. The bacteria produces IAA and can solubilize phosphorus. There are several reports in the literature where IAA production by bacteria has been related to better root development (Loper and Schroth 1986; Noel *et al.,* 1996; Mudeikovsky *et al.,* 1993). Likewise phosphate solubilizing bacteria has been reported to enhance the growth and yield of canola (de Freitas *et al.,* 1997). However, the authors concluded that although P-solubilizing rhizobacteria are potential inoculants for canola, but P-solubilization was not the main mechanism responsible for positive growth response.

At harvest, the straw yield was maximum in plots inoculated with *Klebsiella planticola* with no nitrogen amendment, whereas at nitrogen level of 40 kg N ha^{-1} and 80 kg N ha^{-1}, maximum increases were obtained due to inoculation of *Azospirillum* and *Azotobacter,* respectively. The results clearly indicate the inability of *Klebsiella planticola* to establish and perform its growth promoting activities in the presence of fixed form of nitrogen. Similarly *Azospirillum* could perform well only at lower level of nitrogen (40 kg ha^{-1}). There are several reports in the literature on the poor performance of diazotrophic bacteria and other PGPR at higher levels of nitrogen (Kapulnik *et al.,* 1981; Smith *et al.,* 1984; Tilak and Subba Rao, 1987; Wani, 1988). The presence of fixed form of nitrogen not only represses nitrogenase activity but also leads to catalytic destruction of IAA (Thornton, 1936; Taner and Anderson, 1964; Gibson and Jordan, 1983; Nelson, 1987). Grain yield of a cereal crop is an ultimate criterion to judge the efficacy of an inoculant strain. *Azospirillum* inoculation at 40 kg N ha^{-1} closely followed by *Proteus vulgaris* significantly influenced the grain yield. *Azospirillum* CDJA has been shown to enhance the yield of wheat crop in earlier studies (Kaushik 1998). The strain has the ability to colonize wheat rhizosphere in large numbers, produce IAA in significant amounts (118 µg mg^{-1} protein) at low temperature which prevails during the growing period of wheat.

In other study using the same strains of *Azospirillum, Bacillus subtilis, Klebsiella planticola* and *Proteus vulgaris*, maximum grain yield was reported due to inoculation of *Bacillus subtilis* followed by *Azospirillum* and *Proteus vulgaris* (Singh and Panwar, 1997). However, in their experiment the wheat cultivar used was HD 2428 instead of HD 2285 used in the present study and the crop was raised without any fertilizer amendment. The comparison of the present study with that of Singh and Panwar (1997) brings out certain facts that (a) P-solubilizing activity is more in the absence of available P and hence *B. subtilis* performed well in their study; (b) *Azospirillum* is a better inoculant for wheat and can perform well at low levels of N (40 kg) and recommended dose of phosphorus (80 kg P_2O_5 ha^{-1}); (c) *Proteus vulgaris* has a potential to be used as an inoculant for wheat but needs to be further worked upon. Moreover its coinoculation with *Azospirillum brasilense* would be a worth while treatment to be further tested in the field and (d) Specificity of PGPR strains for a host cultivar do exist. This has been conclusively shown in earlier studies with *Azospirillum* CDJA which showed preference to cultivar HD 2285 as against WH 547 (Kaushik, 1999).

5.3.1.2 Teff

Teff (*Eragrostis tef*) crop is one of the endemic species and unique to Ethiopia. While teff grain still provides over two-thirds of the human nutrition in Ethiopia, it is relatively unknown as a food crop elsewhere. Stallknecht *et al.* (1993) stated that teff grain as being marketed as a health food product, or used as a late planted emergency forage for livestock. It is cultivated in the largest proportion of agricultural land and adapted to a wide range of enviromental conditions. It grows well in a variety of soil types and wide range of altitude (1000-2500 m.a.s.l.) endowed to the country, Ethiopia. However, the yield remains very low, not more than a tonne per hectare despite its hardiness and tolerance to environmental stresses. Information regarding inoculation of PGPR isolates on this crop is not available. Therefore, a pot experiment was conducted with Teff cv. DZ-01-354, in New Delhi, India in order to test its response to different plant growth promoting rhizobacteria.

The investigation showed that PGPR isolates such as *Bacillus subtilis, Klebsiella planticola, Proteus vulgaris* and *Azotobacter chroococcum* increased teff plant development by affecting its different parts. *B. subtilis* and *K. planticola* positively affected teff's height, head size, straw and seed yield while *Azospirillium brasilense* (CDTA) markedly affected the plant height and head size but not straw and seed yield when they are applied without N amendment. This was also true for the others like *P. vulgaris* and *A. chroococcum* (Table 10). However, inoculation of *B. subtilis* and *K. planticola* on teff could be beneficial for plant improvement. It was also clear that nitrogen application delayed crop maturity and as a result the crop was also become succulent and susceptible to pests attack and thereby lowered the yield.

5.3.2 Leguminous Crops

Rhizospheric bacteria of the genera *Rhizobium, Bradyrhizobium, Mesorhizobium, Azorhizobium, Sinorhizobium* can interact with roots of legumes to form nodules. Specific microorganisms have considerable potential to alter the composition and activity of rhizosphere microflora such as *Rhizobium* (Schroth and Ole Becker 1990). Bacteria that promote rhizobacteria (NPR) (Kloepper *et al.*, 1988). Nodulation promoting bacteria belonging to the *Azospirillum* (Schmidt *et al.*, 1988), Pseudomonas (Bottom *et al.*, 1990); Streptomyces (Li and Alexander, 1990); *Bacillus (Halverson and Handelsman, 1991);* Enterobacter *(Gupta* et al., 1998b) genera have been reported. The beneficial effects of these bacteria have been variously attributed to their ability to produce various compounds, including phytohormones (Schmidt *et al.*, 1988; Srinivasan *et al.*, 1996), toxins (Knight and Langston Unkefer 1988), antibiotics (Li and Alexander, 1990), flavonoid like substances (Singh and Gaur, 1995) to suppress deleterious rhizobacteria (Turner and Backman, 1991), or through some other unidentified mechanism (Halverson and Handelsman, 1991). In the present investigation, the three PGPR strains were tested on four leguminous crops namely chickpea, lentil, soybean and mungbean to look for their ability to enhance nodulation, growth and yield of these crops. The results showed that *Proteus vulgaris* in combination with specific *Rhizobium* strain gave maximum nodule number and biomass for mungbean and lentil; while *Bacillus subtilis* for chickpea and soybean gave the highest nodule number and biomass. (Tables 11 and 17). *Proteus vulgaris* could produce IAA, solubilize phosphorus and inhibit soil borne pathogenic fungi while *Bacillus subtilis* and *K. planticola* could solubilize phosphorus and inhibit some of the pathogenic fungi tested (Table 6). Production of IAA enhances root hair formation (Srinivasan *et al.*, 1996). Since root hairs are infection sites for rhizobia, it is possible that the nodulation enhancement observed for lentil and mungbean in response to *P. vulgaris* may be attributable to increased numbers of infection sites available for rhizobia. Zhang *et al.* (1997) also reported that different *Serratia* strains at different root zone reported enhanced number of nodules and acetylene reduction activity in white clovers coinoculated with *R. leguminosarum* bv. *trifolii* and *Azospirillum lipoferum*. They further suggested that the formation of additional infection sites by *A. lipoferum* may be the mechanism that will enhance nodulation and N_2 fixation of white clover.

The phosphate solubilizing rhizobacterial isolates *B. subtilis* and *K. planticola* enhanced the nodulation of chickpea and soybean. Enhancement in nodule number and biomass for soybean has been reported due to coinoculation of *Bradyrhizobium* and phosphate solubilizing microorganism (PSM) (Singh and Singh, 1993). Phosphorus is required in large amounts for better nodulation and PSM provide the legumes with a continuous supply of available phosphorus thereby enhancing the nodulation and growth of legumes.

In general, no significant increase in plant biomass and grain yield was obtained due to inoculation of PGPR singly or in combination with specific rhizobial strains. Dual inoculation of PGPR with *B. japonicum* did not cause any significant effect on plant growth (Singh, 1994). On the other hand, Galal (1997) reported that dual inoculation of *B. japonicum* and *A. brasilense* in sterile and non sterile soils was superior in increasing the dry mass and N-content of the soybean. The results of the present study suggests that stimulation of nodule number alone does not contribute to the enhancement of plant growth and yield under field conditions. One of the reasons could be the occupancy of majority of the nodules with the indigenous population of rhizobia which are mostly ineffective (Sindhu and Dadarwal, 2000). The results obtained on nodule number due to in mungbean, single inoculation of *Proteus vulgaris* and *Bacillus subtilis* gave significantly higher nodule number as compared to uninoculated control (Table 15). This suggests that these rhizobacteria triggers the native population of rhizobia to occupy infection sites and form more number of nodules. Thus there is a need to develop specific combinations of PGPR and *Rhizobium* strains which when coinoculated under field conditions, stimulates the inoculated *Rhizobium* strain to occupy maximum number of nodules induced. The study of Gupta *et al.* (1998b) supports this statement. They found that out of the four PGPR tested only one *Enterobacter* isolate EG-ER-2 could increase the nodule occupancy of *Bradyrhizobium* strain S24. Whereas another isolate KG-ER-1 was found to increase the nodule occupancy of bradyrhizobial strain COG 15 from 77% to 88%.

5.3.3 Oil Seed Crops

Two oil seed crops, mustard and sunflower were used in the present study to evaluate the influence of PGPR isolates. For both the crops, inoculation of these PGPR isolates did not significantly increased the growth and yield. However, in an earlier field study with sunflower, increase in yield from 34% to 56% was reported due to bacterization with *B. subtilis* and *P. vulgaris* (Malik *et al.,* 1996). The inconsistency in results due to inoculation of PGPR strains has been reported and reviewed earlier (Okon and LabanderGonzalez 1994). The main problems impeding a more intensive and widespread use of PGPR have been due to the so called inconsistent results in field experiments. The inconsistency of the response of amended crops to bacterial inoculants could be explained due to given experimental site. Field trials using *Azospirillum* inocula can be presented as a showcase : evaluation of the data collected over the last 20 years demonstrates that in 60 to 70% of the trials, statistically significant increases in yield in the order of 5 to 30% were obtained (Okon and Labandera-Gonzalez, 1994).

The results of the evaluation of these PGPR strains suggest that beneficial traits such as production of IAA and siderophore, phosphate solubilization, inhibition of soil borne fungi and others, used for selection of PGPR isolates under *in vitro* conditioins are only an aid to selection of PGP'R strains from a large number of rhizobacterial isolates. The mere presence of one or all of these traits does not qualify the bacteria to be a PGPR for one particular crop or spectrum of crops. Cottelan *et al.* (1999) reported one isolate which did not share any of the PGPR traits tested *in vitro* except antagonism to *Sclerotium rolfsii* and *Sclerotinia sclerotiorum,* also promoted soybean growth. Moreover, there are reports where exogenous application of IAA or cytokinin or supernatant of PGPR isolates have not brought about a comparable increase in growth as in the presence of rhizobacteria (Lazarovits and Nowak 1997). This indicates that besides the traits mentioned above, there are unexplained mechanisms which also influence the growth of plant and needs the proximity of PGPR to the roots or plants. Another important reason for the inconsistency could be non-optimization of optimal number of cells to be delivered on the seed surface so as to bring the desired effect. Higher or lower dosage of PGPR can have detrimental effect on nodulation and growth of plant as demonstrated by Plazinski and Rolfe (1985 a,b,c). Moreover, PGPR although considered non-specific for crop plants, do show specificity not only for crop plant but also to genotype. The results of the present investigation are a proof to it. The three PGPR worked well for crops like wheat and teff, improved nodulation of leguminous crops but showed no effect on sunflower and mustard. Thus a detailed investigation is required before recommending the use of PGPR strain for a specific crop(s).

6.0 SUMMARY AND CONCLUSION

In the present investigation three newly isolated rhizospheric bacteria, namely *Bacillus subtilis, Klebsiella planticola* and *Proteus vulgaris vis-a-vis* others released PGPR isolates of *Azotobacter chroococcum, Azospirillum brasilense, Bacillus polymyxa, Rhizobium leguminosarum* and *Bradyrhizobium japonicum* were tested under field and greenhouse conditions on several different crops in order to assess their efficiency on crop productivity and specificity.

The three PGPR isolates were also tested for their biochemical reactions and shelf life.

Investigations on biochemical reactions showed that the newly isolated PGPR isolates possess an enormous valuable characteristics that enable them perform well under *in vitro* or *in vivo* conditions. Salient findings of the present investigation are as follows :

- All the PGPR strains were grown in four different media, namely nitrogen free malate medium with bromothymol blue, Pikovskaya medium, nutrient agar and sodium succinate medium.
- Each culture showed distinct growth on different media. All the isolates were found to be positive for citrate utilization, nitrate reduction, ammonia and H_2S production.
- They were able to grow on wide range of C sources. *Bacillus subtilis* on mannitol, *Klebsiella* on lactose and maleic acid and *P. vulgaris* on lactose were not able to grow.

- Intrinsic antibiotic resistance pattern showed variation among the three isolates.
- All the three PGPR isolates could be distinguished from each other through their resistance to varying concentration of trimethoprin, nalidixic acid and tetracycline.
- None of the isolates could fix nitrogen as revealed through acetylene reduction assay.
- All the three isolates could solubilize tricalcium phosphate to varying levels, maximum being by *K. planticola* after 8 days of incubation.
- Of the three isolates, only *P. vulgaris* was found to produce IAA in small amounts. Siderophore production was observed for *K. planticola* and *P. vulgaris*.
- *In vitro* antifungal assay using five different soil borne pathogenic fungi revealed that all PGPR isolates have a potential to inhibit fungal species tested.
- With an objective to develop a delivery system for these PGPR, carrier-based inoculants were prepared using charcoal : soil (3:1) as a carrier. The three isolates could survive for 6 months in the carrier but their shelf life was only for three months.
- Three PGPR isolates and two already recommended biofertilizers (*Azospirillum brasilense,* CDJA; and *Azotobacter chroococcum,* CBD15) were tested on two cereal crops, wheat and teff, for their ability to influence the growth and yield in presence and absence of applied nitrogen. For wheat, maximum grain yield was obtained due to *A. brasilense* (CDJA) inoculation along with 40

kg N ha^{-1}. It was closely followed by treatment inoculated with *P. vulgaris* at the same N level. The results further revealed that in general, with the increase in nitrogen level there was an increase in the population of inoculant bacteria.

- In case of Teff, inocualtion of *Azotobacter* CBD 15 recorded the maximum head size. The biomass and seed yield was not influenced significantly by application of nitrogen fertilizer. In the presence of N fertilizer, inoculation of *P. vulgaris* resulted in maximum seed yield.
- The three PGPR isolates were tested individually and in combination with specific rhizobial strains on four different pulse crops.
- Field inoculation trial of soybean showed that maximum nodule number and mass was obtained with single inoculation of *B. subtilis* followed by *Bradyrhizobium*. Among the coinoculation treatment combination of *Bradyrhizobium* and *P. vulgaris* produced the maximum nodule number and nodule mass. Inoculated treatments did not differ significantly from the uninoculated control with respect to root and shoot dry biomass. Among the single inoculated treatments, *K. planticola* gave the maximum root and shoot dry biomass. However, inoculation with *B. japonicum, P. vulgaris* performed the best. Maximum increment of 19 per cent soybean grain yield and N content were obtained with a combination of *Bradyrhizobium* and *B. subtilis.*
- Maximum lentil nodule number and mass was obtained with a combination of *Rhizobium* and *P. vulgaris* inoculation followed by

dual inoculation of *Rhizobium* and *B. subtilis*. The root and shoot dry weights of lentil plants showed no varations as a result of bacterial inoculation. The highest values for shoot and root dry weight, was obtained due to inoculation of *Rhizobium* and *P. vulgaris*. None of the PGPR isolates either singly or in coinoculation with *Rhizobium* strain could significantly influence the growth and yield of lentil crop.

- The results of mungbean cv. Pusa 9027 showed maximum increase in the number of nodules due to *Rhizobium* strain M11, single inoculations of PGPR isolates except *K. planticola* and dual inoculation of all PGPR isolates with M11. Maximum nodule number, shoot biomass was obtained with dual inoculation of M11 and *B. subtilis.* while highest percentage increment of 16% mungbean grain yield was obtained with *B. subtilis* inoculation followed by 5.7 per cent increment obtained with inoculation of *P. vulgaris*.

- Combination of *B. subtilis* and *Rhizobium* strain F75 inoculants produced the maximum root biomass, nodule number of chickpea cv. Pusa 256. But the accumulation of shoot biomass was depressed. The nitrogenase activity was superior with coinoculation of F75 with the three PGPR isolates although statistically insignificant when compared to single inoculation of PGPR isolates and *Rhizobium* strain. Maximum grain yield increment of 14.3 per cent was obtained due to single inoculation of *Rhizobium* strain F75 followeld by 11.3 per cent by single inoculation of *P. vulgaris*.

- All the PGPR isolates could not significantly increase the growth and yield of two oil seed crops namely mustard and sunflower.

The results of the evaluation of these PGPR strains suggest that beneficial traits such as production of IAA and siderophore, phosphate solubilization, inhibition of soil borne fungi and others, used for selection of PGPR isolates under *in vitro* conditions are only an aid to selection of PGPR strains from a large number of rhizobacterial isolates. There are unexplained mechanisms which also influence the growth of plant and needs further investigation.

The results of the present investigation reveal PGPR although considered non-specific for crop plants do show specificity not only for the host but also to genotype.

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* Original not seen.