BIOCHEMICAL CHARACTERIZATION OF PGPR OF SOYBEAN (*Glycine max* (L) Merill)

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
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By

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Enrolment Number- II/1972 **2012**

DECLARATION OF STUDENT

I hereby declare that, the experimental work and its interpretation of the thesis entitled, **"BIOCHEMICAL CHARACTERIZATION OF PGPR OF SOYBEAN (*Glycine max*(L) Merill)"** or part thereof has neither been submitted for any other degree or diploma of any University, nor have the data been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged

Manoj.

Place : Nagpur
Date: 31/05/2012

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CERTIFICATE

This is to certify that thesis entitled **"BIOCHEMICAL CHARACTERIZATION OF PGPR OF SOYBEAN (*Glycine max*(L) Merill)"** submitted in partial fulfilment of the requirement for the degree of **"Master of Science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Kulkarni Manoj Keshav** under my guidance and supervision.

The subject of the thesis has been approved by the Student's Advisory Committee.

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THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE
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Manoj.

Kulkarni Manoj Keshav

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(D) Abbreviations

Agril.	-	Agriculture
CD	-	Critical Difference
CFU	-	Colony forming unit
cm	-	Centimeter
Conc.	-	Concentration
DAI	-	Days after inoculation
^0C	-	Degree celcious
et al.	-	And co-workers
Fig.	-	Figure
FYM	-	Farm Yard Manure
g/gm	-	Gram(s)
ha	-	Hectare
i.e.	-	That is
J.	-	Journal
Kg	-	Kilo gram
m^2	-	Meter square
NS	-	Non significant
No./no.	-	Number(s)
PDA	-	Potato dextrose agar
PDB	-	Potato dextrose broth
PDI	-	Per cent disease intensity
PDC	-	Per cent disease control
PIY	-	Per cent increase in yield
R	-	Replication
RDF	-	Recommended dose of fertilizer
RH	-	Relative humidity
SE (m) ±	-	Standard error of mean
Sig.	-	Significant
Sr./sr.	-	Serial
T	-	Treatment
Temp.	-	Temperature

Univ.	-	University
Unpub.	-	Unpublished
Viz.	-	Videlicet (namely)
%	-	Per cent
@	-	At the rate of
/	-	Per
µ	-	Micron
C+C	-	Cellobiose+Collistin
Mg	-	Milligram
NA	-	Nutrient Agar
Lit.	-	1 liture
ml	-	Mili liture

(E) THESIS ABSTRACT

a)	Title of the thesis	:	**"BIOCHEMICAL CHARACTERIZATION OF PGPR OF SOYBEAN (*Glycine max* L. Merill)"**
b)	Full name of student	:	**Kulkarni Manoj Keshav**
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ABSTRACT

An investigation entitled "Biochemical characterization of PGPR of soybean (*Glycine max* (L) Merill)" was conducted to characterize the PGPR from rhizosphere of soybean and test

antagonisms against selected soil borne pathogens at Plant Pathology Section, College of Agriculture, Nagpur *in vitro* during the year 2011-12. Plant growth promoting rhizobacteria were isolated from the rhizosphere of soybean plants by using King's B medium and *Enterobacter* on Eosin methylene blue agar medium. The population of *Pseudomonas* were more as compared to *Enterobacter*. Morphological and biochemical characters viz. gram reaction, cell shape, H_2S production, gelatine liquefaction, starch hydrolysis, pyocynin test, fluorescence test, mobility, oxidase test and KOH test were performed. The *Pseudomonas* isolates designated as Pf1, Pf2, Pf3 and Pf4 were gram –ve and rod shaped. All these isolates exhibited +ve test for biochemical analysis, four *Enterobacter* designated as A, B, C and D were gram –ve and rod shaped, mobility and siderophore were having +ve tests. All the *Pseudomonas* isolates showed phosphate solubilization activity and IAA production. Antagonism activities of PGPR were tested against *Fusarium oxysporum* f. sp. ciceri, *Rhizoctonia solani, Sclerotium rolfsii.* The entire PGPR isolate, showed enhancement in germination, shoot and root length and vigour index as compared to control.

Chapter I
INTRODUCTION

1.1 Background Information

Soybean (*Glycine max* (L.) Merrill) belongs to family Leguminaceae. It is unique crop of versatile nutritional attribute yielding in both oil and protein. Soybean other way called 'Golden bean' is the most likely solution for overcoming world protein hunger. Soybean rank first among oil seed crop in the world.

Soybean has its manifold importance in agriculture, medicinal and industrial sector. It has nutritive value. It contains 40-44 per cent protein, 18-20 per cent oil, 30 per cent carbohydrates, and 5 per cent fiber. In addition to this it also contains vitamins A, B, C, D, E, K and all other essential amino acids. Soybean meal or soybean cake resulting from oil extraction processes is used in feeding livestock, poultry, and household pets. Soybean due to its various uses is called 'Wonder Crop' and 'Golden Gift' of nature to the mankind. Soybean is believed to be originated from North Central province of China and it is introduced in India during 1800 A.D. Soybean is cultivated all over the world like USA, Brazil, Argentina and Russia. Among the countries USA occupied 1st rank in area as well as production. India ranks 5th in the world. (Anonymus, 2009).

Plant growth promoting rhizobacteria (PGPR) are group of soil bacteria that actively colonize plant roots and increase plant growth and yield. PGPR belong to a range of genera including *Pseudomonas, Azotobacter, Azospirillum, Bacillus* etc. The mechanisms by which PGPR can promote plant growth are not fully understood, but are thought to include: symbiotic nitrogen fixation (Dobbelare *et al.*, 2003) the ability to produce

phytoharmones (Egamberdieva, 2007) solubilization of phosphate (Catellan *et al.,* 1999) and production of ACC deaminase (Patten and Glick, 2002). Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been demonstrated by many researchers (Asghar *et al.*, 2002, Bashan *et al.*, 2004, Biswas *et al.*, 2000).

In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsilla, Enterobacter, Alcaligens, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have reported to enhance plant growth.(Glick, 1995, Kloepper *et al.*, 1989, Misko and Germida, 2002).

Occurrence of species of *Pseudomonas* in the rhizosphere of different crop plants have been previously reported. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* were particularly effective in increasing root and shoot elongation in canola, lettuce and tomato and yield of potato, radish, sugar beet, tomato, lettuce, apple, citrus, bean, ornamental plants and wheat. (Rodriguez and Fraga, 1999). Several reports indicating that *Pseudomonas* is dominant in the rhizosphere of rice and their inoculation can increase growth and yield production in different parts of the world. (Mahmoud Reza Ramezanpour *et al.*, 2010).

Among the plant growth micro-organisms which are capable to produce herbal harmones we can point to *Azotobacter, Pseudomonas, Azospirillum, Rhizobium, Bacillus, and Enthrobacter* and mycorrhize fungus. Misko and Germida 2002, reported through an experiment that the *Pseudomonas* is the most abundant auxin producer micro-organism. Growth regulators especially IAA (Indole -3- Acetic Acid), often affects the root systematic features such as root primary growth, side-

root formation and root hairs. Auxins are group of herbal harmones which IAA is the most important of them IAA is a natural Auxin with vast physiological effects which plays an important role in growth, increasing and distinction. (Khakipour *et al.*, 2008).

There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activities and metabolism which known as rhizosphere. The rhizosphere concept was introduced by Hiltner (1904) to describe the narrow zone of soil surrounding the roots, where microbe populations are stimulated by root activities. A large number of micro-organisms such as bacteria, fungi, protozoa, and algae coexist in the rhizosphere. Bacteria are the most abundant among them.

1.2 Importance of study

Soil borne fungal pathogens are major threats to cereals and pulses which are responsible for heavy yield losses annually, Chickpea wilt caused by *Fusarium oxysporum* f. sp. ciceri accounts for 10-100% yield losses annually in India depending on varietal susceptibility and agroclimatic condition. (Chand and Khirbat, 2009). *Sclerotium rolfsii* is responsible for 25% yield losses in groundnut. (Mayee and Datar, 1988). Plant losses up to 77% and yield losses 30-35% reported in soybean crop due to *Rhizoctonia bataticola* (Muthusamy and Mariappan, 1991).

Spraying, drenching, seed treatment and soil application of fungicides can control these pathogens but these are not economical solution and are polluting agents of the environment. Frequent application of fungicides may lead to the development of tolerance in the target organisms. Hence biological control of plant diseases using antagonistic bacteria (*Pseudomonas*

fluorescens) may be considered as a promising alternative to the use of some hazardous chemical fungicides.

Biological control is gaining momentum as an essential component. Amongst biological agents, at present P. *fluorescens* is receiving much attention. These bacteria have an ability to rapidly colonize the roots of host plants and thus compete with the soil borne pathogens for space and food, keeping in mind, present research work was undertaken entitled "Biochemical characterization of PGPR of soybean (*Glycine max*) with a objective to isolate and characterize the native strains of P. *fluorescens* from rhizosphere of soybean and to find out efficient strain of P. *fluorescens* against soil borne fungal pathogens.

1.3 Objectives

1. Characterization of rhizobacteria isolates.
2. Antagonism against selected soil borne pathogens.

1.4 Hypothesis

Plant growth promoting rhizobacteria and bioagents have good prospectus in future as they give very high cost benefit ratio In view of this 1st assumption is to isolate the P. *fluorescens* bacteria from the various field crop rhizosphere with maximum antagonistic activity against soil borne fungal pathogens under Indian environmental conditions and to determine the ability of selected bacterial isolates to suppress the soil borne fungal pathogens under *in vitro* condition.

One of the mechanisms of P. *fluorescens* is their ability to produce siderophores for sequestering iron. The secreted siderophores binds to the Fe^{3+} that is available in rhizosphere and thereby effectively prevent growth of pathogens in that region, this information will be helpful to study antibiosis of P.

fluorescens against *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani*.

1.5 Scope and limitation

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria i.e. P. *fluorescens* to increase plant growth has shown considerable promise in laboratory and green house studies. The potential environmental benefits of this approach leading to reduction in the use of agricultural chemicals and fit with sustainable management practices.

We can expect to see new P. *fluorescens* products becoming available to farmers as a biofungicides. The success of these products will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial micro-organisms. Sequencing the genome provided further information of its environmental interaction and its metabolic capabilities, which can be used to control plant diseases. Though P. *fluorescens* is most widely used bicontrol agents, but the major limitation of these biocontrol agents is not only their shelf life but also in consistent field performance. (Zaidi *et al.*, 2004).

Unlike chemical pesticide, biocontrol agents need support even after their application to get established in targeted niche. Therefore the success of biological control, one has to ensure not only the quality of biocontrol agent application but also their establishment in natural ecosystem to thrive and compete well with the pathogens of other microbes. Development of better formulations to ensure survival of activity in the field and compatibility with chemical & biological seed treatments is another area of focus.

Chapter II

REVIEW OF LITERATURE

Studies regarding the "Biochemical characterization of PGPR of soybean. (*Glycine max* L. Merill)" were taken up.

In this study plant growth promoting Rhizobacteria (PGPR) *Pseudomonas fluorescence* were isolated from rhizosphere of soybean (*Glycine max*. L. Merill).

All the isolates were characterized on the basis of Morphological biochemical and molecular characterization.

All the isolates were tested for plant growth promoting activities viz. Siderophore production, phosphate solubilization, Auxin (IAA) production and *in vitro* efficacy of isolates for antibiosis against selected soil borne pathogens i.e. *Fusarium oxysporum* f. sp. ciceri, *Sclerotium rolfsii, Rhizoctonia solani.*

Attempts therefore, have been made to put forth the partient recently published work reviewed critically under the following major heads.

2.1 Isolation of plant growth promoting rhizobacteria
2.2 Rhizospheric population of PGPR
2.3 Morphological and biochemical characteristics of PGPR
2.4 Growth pattern of P. *fluorescens* on different media
2.5 Siderophore production
2.6 Phosphate solubilization activity of PGPR
2.7 *In vitro* antibiosis of PGPR (P. *fluorescens*) against soil borne fungal pathogens.

2.1 Isolation of plant growth promoting rhizobacteria

Jayswal *et al.* (1990) isolated the *Pseudomonas* strain from the caryopsis of grass *Tripsacum dactyloids* and was identified as *Pseudomonas cepacia.* The isolated *Pseudomonas* strain showed antagonism against *Aspergillus flavous, Fusarium moniliforme and Rhizopus stolonifer* on PDA medium.

Baig *et al.* (2002) isolated the plant growth promoting rhizobacteria from rhizosphere of groundnut by serial dilution technique and identified up to generic level. Two isolates identified as *Enterobacter spp.* Which promoted highest plant growth followed by one isolated each of *Pseudomonas fluorescens* and *Bacillus spp.* in the green house pot trial and field trial.

Husen (2003) tested the fourteen isolates of soil bacteria, two known plant growth promoting rhizobacteria (PGPR) *in vitro.* Phosphate solubilization and siderophore production were tested qualitatively by plating the bacteria in pikovaskayas and Chrome azurole S agar. The results showed that twelve isolates produced IAA, ability to solubilize phosphate positively exhibited by 4 isolates. Seven isolates produced siderophore.

Garima Jha *et al.* (2005) studied the isolation of PGPR from the rhizosphere of *Vigna radiata, Arachis hypogea* and *Abrus ptionctoris* by using double layer technique (DLT) and showed antagonism to potentially deleterious rhizoplane fungal and bacterial pathogens such as *Rhizoctonia solani, Fusarium oxysporum and Sclerotium rolfsii.* Seed bacterization of tomato resulted in growth promotion of plants.

Khakipour *et al.* (2008) isolated the strains of fluorescent *Pseudomonas* from Iran soils and evaluated about secretion of

Auxin compounds. The amount of exuded IAA by fluorescent *Pseudomonas* strains was varied from zero to 31.6 mg/l while it was producing from zero to 24.08 mg/l in P. *putida*.

Hynes *et al.* (2008) studied 563 bacteria originating from the roots of pea, lentil and chickpea grown in Saskatchewan and screened for several plant growths promoting traits viz. suppression of legume fungal pathogens and for plant growth promotion. Out of them 76% isolates positive for siderophore production, 5% suppressed the growth of *Pythium spp.*, 5% amino cyclopropane-1-carboxylic acid (ACC) deaminase activity, 7% Indole product, 7% suppressed the growth of F. *avenaceum* and 9% suppressed the growth of *Rhizoctonia solani*. The isolates were positive to plant growth promotion and belonging to the members of Pseudomanadaceae and Enterobacteraceae family.

Chaiharn *et al.* (2008) screened rhizobacteria comprising of 220 phosphate solubilizing bacteria from different rhizosphere for production of ammonia, siderophore and all were found degrading enzymic activities, cellulose, chitinase and proteolytic enzymes. 64% screened rhizobacteria ammonia, 23% produced siderophore were studied. The results showed that rhizospheric phosphate utilizing bacteria could be a promising source for growth promoting agent.

Sarode *et al.* (2009) isolated the bacteria from the wheat rhizosphere in black cotton soils of North Maharashtra region and tested subsequently for *in-vitro* for siderophore production. Wheat isolate SCWL being a strong siderophore producer was selected, identified and confirmed as *Acinetobacter calcoceticus*. The strain produced catechol type of siderophores during exponential phase, which was influenced by iron content of medium siderophore mediated antagonism was observed against

common phytopathogens viz. *Aspergillus flavous*, *A. niger*, *Colletotrichum capsicum* and *Fusarium oxysporum*.

Gholami *et al.* (2009) inoculated the plant growth promoting rhizobacteria on maize seed using six bacterial strains viz. *P. putida* strain R-168, *P. fluorescens* strain R-93, *P. fluorescens* –DSM 50090, *P. Putida* DSM 291, *A. lipoferum* DSM 1691, *A. brasilense* DSM 1690. The results revealed that seed inoculation significantly enhanced seed germination and seedling vigour of maize. Strains also increased leaf and shoot dry weight and leaf surface area were significantly increased due to bacterial inoculation in both sterile and non sterile soil.

Naz *et al.* (2009) examined plant growth promoting rhizobacteria from rhizosphere of four weeds viz. *chrysopagon aucheri, Lactucca dissecta, Solanum surratense* and *Sonchus arvensis*. It was revealed from the experiments that all the strains were capable to produce phytoharmones IAA, Gibberellic acid (GA3), Trans zeatin ribose (t-zr) and Abscicic acid (ABA) in culture media.

Yeon and Kyung-Suk (2009) isolated the plant growth promoting rhizobacterium *Serratia sp.* SY5 from rhizoplane of barnyard grass. These isolate has shown capacities for Indole acetic acid production and siderophore synthesis.

Battu and Reddy (2009) isolated and characterized as *Pseudomonas fluorescens* strains from rice growing soil samples. The isolates tested against *Rhizoctonia solani* shows inhibition of up to 69.8% as compare to control.

Bhromsiri and Bhromsiri (2010) evaluated the 136 isolates of rhizobacteria from rhizosphere of different rice varieties, and determined their acetylene reduction activities and Indole acetic

acid production activities. The amount of exuded IAA by rhizobacteria strains was varied from 0.05 to 99.59 mg^{-1}.

Mahmoud *et al.* (2010) isolated the fluorescent *Pseudomonades* from rhizosphere of rice (*Oryza sativa* L. in specific) of different provinces in northern Iran and characterized by standard morphological and biochemical methods. Those isolates were tested to identify the production of Indole acetic Acid (IAA). The IAA production in presence of tryptophan (50 mgL^{-1}) in the strain was recorded within the range of 17.7-95.9 $\mu g\ mL^{-1}$.

Maleki *et al.* (2010) isolated the *Pseudomonas fluorescens* plant growth promoting rhizobacteria (PGPR) from cucumber rhizosphere. The green house studies shows the highest colonization on the roots and significantly promoted plant growth *in vitro* condition. These isolates produced siderophore and Indole acetic acid (IAA) production with addition of tryptophan.

Saharan and Nehra (2011) stated that inoculation of ornamentals, forest trees, vegetables and agricultural crops with PGPR enhanced seedling germination, stand health, plant vigour, plant height, shoot weight, nutrient content of shoot tissues. PGPR reported to influence the growth, yield and nutrient uptake by an array of mechanisms. It helps in increasing supply of phosphrous, sulpher, iron and copper, produce plant hormones, enhance other beneficial bacteria or fungi, control fungal and bacterial diseases and helps in controlling insect pests.

Carlos *et al.* (2011) isolated the 97 isolates. Out of them 18 isolate produced IAA, 27 were able to solubilize organic phosphate, 37 were positive for siderophores and 45 antagonistic

to *Fusarium oxysporum*. The isolates were belonging to Baciliaceae, Enterobacteraceae and Pseudomanadaceae families.

Nathan *et al.* (2011) isolated the plant growth promoting rhizobacteria (PGPR) *Pseudomonas fluorescens* from rhizosphere of rice and characterized by testing various biochemical test. These isolates showed positive for catalase and oxidase activity, motility, and growth at 4°C confirming them to be *Pseudomonas spp.*

2.2 Rhizospheric population of PGPR

Bhatia *et al.* (2005) studied on colonization of sunflower roots by *fluorescent Pseudomonas* and stated that rhizospheric population of *fluorescent Pseudomonas* strains increased in rhizosphere up to 66 days of sowing but slightly decreased thereafter. One gram rhizospheric soil contained 5.5 -7.5 x 10^5 cfu bacterial population.

Siddiqui and Shakeel (2009) studied 21 isolates of *Pseudomonas* which colonized pigeon pea roots. Maximum colonization was of Pf 737 followed by Pf 736 and Pf 740. The root colonization by different *Pseudomonas* isolates were between 2.3 x 10^4 to 1.2 x 10^4 cfu/g of soil.

2.3 Morphological and biochemical characteristics of PGPR

Hegedoron (2001) identified characteristics of PGPR which were strictly straight or curved rods, without spore, cells stain gram negative, chemo organotrophic, catalase positive, colonies were unusually white to cream to yellow pigmented, many species produce extra cellular fluorescent pigment.

Shinde (2003) reported that among ten *P. fluorescens* isolates, seven produced H_2S gas, eight isolates showed hydrolysis of starch, nine liquefied the gelatin and nine isolates gave positive test for auxin production (IAA).

Mahesh (2007) investigated physiological and biochemical reaction of ten *P. fluorescens* isolates. All isolates were gram negative and rod shaped. All ten *P. fluorescens* isolates showed variable reaction in case of starch hydrolysis, gelatin liquefaction and H_2S production.

Muzumdar *et al.* (2007) isolated nine *fluorescent Pseudomonas* isolates on King's B medium from rhizosphere of tea plants and compared to strain *P. fluorescens* MTCC – 103 for their biochemical and functional characteristics. It produced IAA like substances, siderophore and soluble P.

Tiwari and Thrimurthy (2007) studied on morphological and biochemical characteristics of seven isolates of *Pseudomonas fluorescens* and found that all the isolates showed positive reaction to gelatin liquefaction, catalase test, oxidase test, ammonification, starch hydrolysis, li reaction in litmus milk and strictly aerobic.

Siddiqui and Shakeel (2009) characterized twenty isolates of *Pseudomonas fluorescens* on the basis of gram reaction, gelatin liquefaction and starch hydrolysis. All the isolates were gram negative, showed negative test for starch hydrolysis and positive test for gelatin liquefaction.

2.4 Growth pattern of P. *fluorescens* on different media

Yeole and Dube (2001) isolated 10 rhizobacterial *Pseudomonas fluorescens* isolates on NA and KB medium and observed that the percentage of *fluorescent Pseudomonas* was more on the KB that on the NA medium

Bhatia *et al.* (2005) performed gram reaction of *Pseudomonas fluorescens* isolates and produced transparent, smooth margin, small colonies with diffusible fluorescent pigments in succinate broth.

Bhosale (2005) studied morphological characteristics of two *Pseudomonas fluorescens* isolates on King's B medium. Isolate P1 showed circular convex shaped colonies with whitish pigmentation while isolate P2 showed circular slightly raised colonies with white to yellowish pigmentation.

Tiwari and Thrimurthy (2007) studied cultural characteristics of seven isolates of *Pseudomonas fluorescens* on King's B medium and observed that the isolates were gram negative and rod shaped that produced round to irregular colonies and with yellowish, dull yellowish and greenish yellowish water soluble pigment production. All the isolates showed bright reaction to UV light and slow as well as fast growth on KB medium.

Siddiqui and Shakeel (2009) observed that green fluorescence of *Pseudomonas spp.* was very clear on King's B medium.

2.5 Siderophore production

Yeole and Dube (2000) obtained twelve (12) rhizobacterial *fluorescent Pseudomonas* isolates from chilli, cotton, groundnut and soybean rhizosphere. All the isolates produced siderophore under iron deficient condition.

Qing and Jian (2011) reported the activities of *Bacillus subtillis* which produced siderophores, a high affinity chelating agents for ferric iron on a simple double – layered Chrome azurole S agar (SD – CASA) plates. It is one of the mechanisms by which bicontrol agent act in inhibiting the growth of phytopathogens.

Bhatia *et al.* (2005) estimated production of siderophore by Chrome Azurole (CAS) assay and stated that appearance of an orange coloured zone after 48 hr. of incubation indicated that the isolated strains of *fluorescent Pseudomonas* were able to chelate Fe^{3+} from chrome azurole sulphonate medium.

Paez *et al.* (2005) studied that *Pseudomonas aeruginosa*, *Pseudomonas putida* biovar B, *Pseudomonas marginalis* and *Burkholderia cepacia* isolated from rhizosphere and phyllosphere of rose, identified by biochemical assays and cultured in King's B medium, showed antagonistic properties, *in vitro*, against the pathogens *Rhizoctonia solani* and *Botrytis cinerea* on PDA medium. These properties coincided with presence of siderophore.

Sayyed *et al.* (2005) studied and showed that the two strains of *Pseudomonas* fluorescens and *Pseudomonas putida* produced siderophores in modified succinic acid medium (SM) and also showed that *Pseudomonas fluorescens* inoculation

enhanced seed germination, root length and shoot length of wheat (*Triticum aestivum*) under pot culture conditions.

Krey (2008) obtained 867 heterotrophic bacterial isolates from five depth profiles inside and outside of the dome and screened for siderophore production using the Chrome Azurole – S (CAS) assay.

Gupta and Murali (2008) showed the plant growth promoting rhizobacteria (PGPR) produced siderophores which was detected by Chrome Azurole S assay.

Bhattacharya (2010) showed *P. fluorescens* produced siderophore pigment and detected by using CAS assay and catechol siderophores by Arnow assay. The experiment results showed that siderophore pigment production was increased in the presence of sodium, potassium, lead, molybdenum and cadmium by *Pseudomonas fluorescens*.

2.6 Phosphate solubilization activity of PGPR

Rodriguez *et al.* (1999) studied the role of phosphate solubilizing bacteria as inoculants and was found increasing P uptake by the plant and their by crop yield. These parameters strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. The principal mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphates play a major role in the mineralization of organic phosphrous in soil.

Vazquez *et al.* (2000) isolated the phosphate solubilizing bacteria from rhizosphere of white and black mangrove. Thirteen phosphate solubilizing bacteria isolated from both species of mangroves containing genera *Bacillus*, *Enterobacter*,

Pseudomonas. The results showed that phosphate solubilizing activity of the isolates was first qualitatively evaluated by the formation of halos (clear zones) around the colonies growing on solid medium containing tri calcium phosphate as a sole phosphrous source.

Sharma *et al.* (2007) isolated two strains of phosphate solubilizing bacteria from soil sample and identified as *Pseudomonas fluorescens* and *Bacillus megaterium* on the basis of their morphological, cultural and biochemical reactions. Their phosphate solubilizing efficiency confirmed on Pikovaskaya's medium. Results suggest that seed treatment with these phosphate solubilizing bacteria enhances seedling length and can be used as a biofertilizer.

Afzal and Bano (2008) examined *Rhizobium* and phosphate solubilizing bacteria *Pseudomonas spp* when inoculated with wheat (*Triticum aestivum*) improved the yield, phosphorous uptake, IAA production and GA production in wheat.

Yazdani Mohammad *et al.* (2009) studied the effect of phosphate solubilization micro-organisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays*). The results showed that in all fertilizer treatment application of PSM and PGPR together could reduced P application by 50% without any significant reduction of grain yield. Furthermore, using of PSM and PGPR in addition to conventional fertilizer applications (NPK) could improve ear weight, row number and grain number per row and ultimately increased grain yield in green manure and check plots.

Sharma *et al.* (2011) isolated the phosphate solubilizing bacteria from rhizosphere of Park, graveyard and identified as

Pseudomonas fluorescens on the basis of biochemical characteristics. Their phosphate solubilizing activity was detected by formation of clear halo zones on pikovaskayas medium having inorganic tricalcium phosphate.

2.7 Antibiosis of PGPR against soil borne fungal pathogens

Geels and Schippers (1983) isolated 112 *fluorescent Pseudomonas* isolates from potato periderm and screened for their antagonistic property. 57% of the isolates showed wide spectrum inhibitory activity, mostly due to the production of fluorescent siderophores.

Expert and Digat (1995) tested the efficacy of *Pseudomonas fluorescens* and *Pseudomonas putida* strains against *Sclerotinia sclerotiorum in vitro* and *vivo*. Significant protection of sunflower was obtained by seed bacterization with selections of *P. fluorescens* and *P. putida*.

Liu *et al.*, (1995) obtained the systemic resistance of plant growth promoting Rhizobacteria (PGPR) against *Fusarium* wilt in cucumber. Plant Growth Promoting Rhizobacteria (PGPR) strains *Pseudomonas putida* and *Serratia marcescens* tested for their ability to induce systemic resistance against *Fusarium* wilt. The results revealed that both PGPR strains induced systemic resistance against *Fusarium oxysporum* f.sp. cucumerinum.

Sindhu and Dadarwal (2001) isolated the *Pseudomonas* strains from the rhizosphere of chickpea (*Cicer arentinum* L.) and green gram (*Vigna radiata* L.) and screened for production of chitinases and cellulases. *Pseudomonas* produced appreciable amounts of both enzymes in culture free supernatants and exhibited growth inhibition of

the two fungi *Pythium aphanidermatum* (oomycete) and *Rhizoctonia solani* (Basidiomycete) in plates on potato dextrose agar medium.

Ramamoorthy *et al.* (2001) studied the systemic induction of resistance by plant growth promoting rhizobacteria (PGPR) in chickpea against pest and diseases. The genera belonged to *Pseudomonas spp*. Seed treatment with PGPR caused cell wall structural modification and biochemical, physiological changes to the synthesis of proteins and chemicals involved in plant defence mechanism. Lipopolysaccharides, siderophore and salicylic acid are the major determinants of PGPR.

Ramamoorthy *et al.* (2002) isolated the *Pseudomonas fluorescens* from the rhizosphere of different crops and identified as *Pseudomonas fluorescens* based on gelatin liquefaction, arginine dihydrolase and nitrate reduction. Among the different isolates tested *P. fluorescens* isolate Pf 1 increased plant vigour and also found to protect tomato plants from wilt disease caused by *Fusarium oxysporum* f. sp. lycopersici.

Siddiqui and Shankat (2002) studied plant growth promoting rhizobacteria (PGPR) *Pseudomonas aeruginosa* (IE-65) and *Pseudomonas fluorescens* (CHAO) and suppressed the growth of damping off of tomato *Rhizoctonia solani.*

Nandkumar *et al.* (2002) studied the antifungal activity of *Pseudomonas fluorescens* strains PF1, PF7, and PB2 and tested against rice sheath blight pathogen *Rhizoctonia solani.* Results showed that the antibiotic produced by bacterial strains 2, 4-diacetyl. Phloroglucinol, iron chelating siderophore, hydrogen cynide, lytic enzymes such as chitinase were correlated with antifungal activity against *Rhizoctonia solani.*

Begum *et al.* (2003) studied the effectiveness of plant growth promoting rhizobacteria isolates against some seed borne fungal *Fusarium*, seed treatment of okra with PGPR showed increased the biomass of plants, total number of leaves, fruits, mean length, girth and yield of okra. The PGPR treatment reduced most of seed borne fungal pathogens like *Fusarium*, *Rhizoctonia* and enhanced seed germination, vigour, quality and yield of okra.

Paolina *et al.* (2004) studied the antagonistic activity of 500 *Pseudomonas* isolates against *R. solani* AG 3 tested by dual culturing on Potato dextrose agar medium. Results revealed that 17.4% (84 of 500) of all isolates exhibited the ability to suppress the *R. solani* AG3.

Saravanan *et al.* (2004) isolated the strains of *Pseudomonas fluorescens* from rhizosphere of banana. These isolates used for assessing their antifungal efficacy against *F. oxysporum* f.sp. cubense *in vitro*. The results demonstrated that all four strains isolated from the rhizosphere of banana had significant inhibitory action on the growth *F. oxysporum* f.sp. cubense.

Manjula *et al.* (2004) studied combined application of *Pseudomonas fluorescens* and *Trichoderma viride* for their biocontrol activities against stem rot in groundnut caused by *Sclerotium rolfsii*. Four isolates of *Pseudomonas fluorescens*, GB4, GB8, GB10 & GB27 and *T. viride* Pg1 were identified as potent antagonists of *S. rolfsii*. The results of study showed the combined application of either GB10 or BG27 with *T. viride* Pg1 was significantly effective than that with Thriam in protecting groundnut seedlings from stem rot infection caused by *Sclerotium rolfsii*.

Nagrajkumar *et al.* (2004) isolated 14 strains of *Pseudomonas fluorescens* from rhizosphere soil of rice and tested for their antagonistic effect against *Rhizoctonia solani* causing rice sheath

blight. Among them Pf MDU2 was the most effective in inhibiting mycelial growth of *Rhizoctonia solani in vitro*.

Kumar *et al.* (2005) isolated the *fluorescent Pseudomonas* strains from the rhizosphere of matured tomato and characterized then by determining IAA production, siderophore production, and HCN production. The bacterized seed showed significant increased in shoot length, shoot fresh weight and shoot dry weight of tomato. *Pseudomonas* PE10 strain strongly inhibit the growth of *F. oxysporum* on Tryptic 50% agar plates (TSM) at 28 ± 1°C.

Kaur *et al.* (2007) examined the antagonistic activity *in vitro* against growth of *Fusarium oxysporum* f.sp. ciceri in 90 isolates of *fluorescent Pseudomonas* obtained from the rhizosphere of chickpea. Results showed that under green house and field condition, the isolates of *fluorescent Pseudomonas*, significantly enhanced seed germination, reduced disease incidence i.e. inhibit the growth of *Fusarium oxysporum* f.sp. ciceri *in vitro* and promoted plant growth of chickpea as compared to control. .

Rini and Sulochana (2007) tested the antagonistic activity of *Pseudomonas fluorescens* and *Trichoderma viridae* against *Fusarium oxysporum* and *Rhizoctonia solani* infecting tomato. The results of *Pseudomonas fluorescens* (P-28) showed the greatest inhibition against *Rhizoctonia solani* and *Fusarium oxysporum*.

Salam *et al.* (2007) studied the antagonistic activity of *Pseudomonas fluorescens* against *Fusarium oxysporum* and showed the *fluorescent pseudomonas* potentially inhibit the growth of *Fusarium oxysporum*.

Rajeshwari and Kannabiran (2011) studied the antagonistic activity of *Trichoderma viridae*, *Trichoderma harzianum* and *Pseudomonas fluorescens* against *Fusarium oxysporum in vitro*. The result showed highest per cent inhibition of conidial germination by *Trichoderma viridae* (89.4%) followed by *T. harzianum* (85.7%) and *Pseudomonas fluorescens* (83.15%), Antagonistic activity of *Pseudomonas spp* against *Fusarium oxysporum* is mainly due to the antibiotics, Fe chelating siderophores and hydrogen cynide

Chapter III

MATERIAL AND METHODS

The present investigation entitled "Biochemical characterization of PGPR of soybean (*Glycine max* L. Merill)" was carried out in the laboratory of Plant Pathology Section, College of Agriculture, Nagpur.

3.1 Collection of soil samples for isolation of PGPR

Soil samples were collected from rhizosphere of soybean from Agronomy field, Entomology field, Soil Science field, and from Plant Pathology field respectively of College of Agriculture, Nagpur.

3.2 Sterilization of glass wares, media and water

Petriplates, test tubes and conical flask of different capacities i.e. 1000 ml, 500ml, 250 ml of "Borosil" make were used. The glassware's were sterilized in hot air oven at 180°C for one hour. The media and distilled water were sterilized in autoclave at 15 lbs for 30 minutes.

3.3 Precaution to eliminate contamination

All isolation and inoculation work of microbial cultures were carried out in laminar air flow. The laminar airflow was sterilized by glowing ultraviolet lamp 30 minutes prior to commencement of work.

3.4 Isolation of plant growth promoting Rhizobacteria

The plant growth promoting rhizobacteria were isolated from soil samples collected from rhizosphere of soybean (*Glycine*

max) by serial dilution method. For isolation of *Pseudomonas fluorescens* King's B medium was used (King *et al.* 1954).

King's B medium

Peptone	-	20 g
Glycerol	-	10 ml
K_2HPO_4	-	1.5 g
$MgSO_4.7H_2O$	-	1.5 g
Agar –Agar	-	15 g
Distilled water	-	1000 ml
Cyclohexamide	-	1 mg.

All the media were supplemented with antifungal compound Cyclohexamide 1mg to inhibit fungal growth.

Soil samples were collected from rhizosphere of soybean (*Glycine max*). Test tubes with 9 ml distilled water were sterilized in autoclave for preparation of water blank procedure.

1. Soil samples were collected from rhizosphere of soybean from Agronomy, Entomology, Soil Science, and Plant Pathology field College of Agriculture, Nagpur. Mixed and prepared compost samples.

2. Labeled the distilled sterilized water blanks as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 4 plates marked with marking pencil.

3. Prepared initial dilution by adding 1 g of air dried soil sample from composite sample in to 9 ml distilled sterilized water blank and labeled 10^{-1}. Thus diluting original sample 10 times (1:10).

4. Vigorously shaken on a magnetic shaker for 20-30 minutes to obtain uniform distribution and release of micro-organism from adhesing soil particles.

5. Allowed the soil particles to settle and from the 1st dilution transferred 1 ml of suspension to dilution blank number of 10^{-2} by sterile pipette shaken well for a minutes.

6. From 10^{-2} suspension transferred 1 ml of suspension to 10^{-3} dilution blank with a fresh sterile pipette.

7. Repeated the procedure till the original sample had been diluted to 10^{-7} using every time a fresh sterile pipette.

8. From the appropriate dilution (10^{-6} and 10^{-7}) transferred 1 ml of suspension with respective pipette to sterile petriplates. Three petriplates were used for each dilution.

9. Approximately 20 ml melted medium added to each petriplates containing the diluted sample. Mixed the content of each dish by rotating gently to distribute the cell throughout the medium.

10. Allowed the petri plates to solidify and incubate these plates in an inverted position for 2-7 days at 28±2°C. The 6th and 7th dilution were plated in petriplates containing selective medium for isolating *Pseudomonas fluorescens*.

11. Well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light.

12. The individual colonies were picked up with sterilized loop and transferred on fresh King's B medium.

13. The plates were incubated at 28±2°C for 24 hr.

14. The single colonies developed were transferred in King's B medium slants and the pure cultures so obtained were stored in refrigerator at 4°C for further investigation.

3.5 Counting of Rhizospheric population of *Pseudomonas fluorescens*

Rhizospheric population of *Pseudomonas* was counted by dilution and plate count method. After four days of incubation the total numbers of colonies were counted and calculated the number of micro-organism per volume of original suspension as per following formula.

$$\text{Organism/g of soil sample} = \frac{\text{No. of colonies}}{\text{Amount of Suspension x dilution factor plated}}$$

3.6 Identification of culture

Bacterial cultures were identified by following biochemical tests:

Biochemical studies

Biochemical test viz. H_2S production, gelatin liquefaction, starch hydrolysis, casein hydrolysis and grams reaction was carried out for biochemical confirmation of *P. fluorescens*. All the isolates of PGPR were also evaluated for plant growth promoting properties viz. IAA production and phosphate solubilization.

3.6.1 Gram Reaction
3.6.2 H_2S Production
3.6.3 Gelatin Liquefaction
3.6.4 Starch Hydrolysis
3.6.5 Pyocynin Test
3.6.6 Fluorescence Test
3.6.7 Catalyse Activity
3.6.8 Oxidase Test
3.6.9 KOH Test
3.6.10 Hydrogen sulphide production test
3.6.11 Siderophore Production
3.6.12 Phosphate Solubilizing Activity
3.6.13 IAA Production
3.6.14 *In vitro* antibiosis

3.6.1 Gram's reaction

Gram reaction was carried out for bacterial cultures to classify them in two groups i.e. Gram positive and Gram negative

Procedure

i. Loopful of bacterial suspension was transferred in the centre of slide with the help of wire loop.
ii. The drop was smeared over slide and air dried.
iii. Then dried smear was fixed by passing the slide 3-4times rapidly over the flame.
iv. The smear was flooded with crystal violet for 30 seconds and then washed in the tap water.
v. Then the smear was immersed in potassium iodide / Lugol's iodine solution for 30 seconds washed in tap water then decolorized with 95% alcohol and rinsed with water.
vi. Counterstained with saffranin for 10 second, again washed with tap water and air dried.

vii. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lense.

3.6.2 H_2S Production

The activity of bacterium on sulpher containing amino acids frequency results in production and liberation of H_2S gas.

Medium

Peptone water = peptone 1%, cystine 0.01%

Lead acetate paper was prepared by moistening the filter paper in saturated solution of lead acetate. Lead acetate paper was inserted in the tubes containing peptone water inoculated with bacterium and hold by the plugs above the culture without touching the medium. The tubes were incubated for three days. If filter paper strips turns black, it indicated the positive test for H_2S production.

3.6.3 Gelatin Liquefaction

The test indicated utilization of protein and production of proteolytic enzymes by bacterium.

Medium

Nutrient gelatin = Nutrient broth + 1.5% gelatin

Bacterial culture were inoculated through stab of a nutrient gelatin tube and incubated for seven days uninoculated tubes serves as control and observed for liquefaction.

3.6.4 Starch Hydrolysis

Starch is a complete carbohydrate of the polysaccharide type, hydrolyzed by the bacterium. The positive test indicates by the presence of amylase enzyme utilized for hydrolysis of starch.

Medium

Starch Agar = Nutrient Agar + 0.2% Soluble starch

Test reagent = Lugol's iodine solution

Bacterial culture was inoculated on starch Agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of Starch hydrolysis indicated by the appearance of clear zone. Reddish zone indicated the starch was partially hydrolysed to dextrin.

3.6.5 Pyocynin Test

Material Required

24-48 hrs old bacterial culture
Pseudomonas agar medium
Sterile petriplate

Procedure

Pseudomonas Agar medium

Peptone	- 20 gm
$MgCl_2$	- 1.4 gm
K_2SO_4	- 10 gm
Agar	- 20 gm
Distilled Water	- 1 lit.

Stimulate the pyocynin production prepare Pseudomonas agar medium, autoclave it then melt the agar medium, pour the

medium at 40°C in sterile petriplate and allow it for solidification, when make simple streak across the surface of room temp for 24-48 hrs. after binding to the bacteria. The petriplate is viewed under U.V. light. The presence of dark green or bluish colour was interpreted as indicating the production of pyocynin.

3.6.6 Fluorescence Test

Material required

24-48 hrs old bacterial culture
Pseudomonas agar medium
Sterile petriplate

Procedure

Pseudomonas agar medium

cosel^{n} enzymic hydrolysate -	10 gm
Proteose peptone -	10 gm
K_2HPO_4 -	1.5 gm
$MgSO_4$ -	2.5 gm
Agar -	20gm
Distilled Water -	1000 ml.

Favours the formation of fluorescent prepare Pseudomonas agar F. medium autoclave it then melt the agar medium. Pour the medium at 40°C in sterile petriplate and allow it for solidification. When make simple streak across the surface of medium. Incubate the plate at room temp for 24-48 hrs. After binding to the bacteria. The petriplate is viewed under U.V. light. The presence of yellow pigment only was interpreted as indicating the production of fluorescens.

3.6.7 Catalyse Activity

Nutrient agar tubes were inoculated with bacterial culture and incubated for three days. A bit of growth was removed from the slants and placed on a clean glass slide. 3% H_2O_2 was added on it .Appearance of bubbies showed positive test for catalase.

3.6.8 Oxidase Test

Material Required

24-48 hrs old bacterial culture
Oxidase disc

Procedure

The 24 hrs old bacterial culture were spot inoculated on oxidase disc and change in colour of the disc from white to purple or blue was observed.

3.6.9 KOH Test

Material Required

24-48 hrs. Old bacterial culture
3% KOH solution
Cavity slide
Inoculation needle

Procedure

A loopful of bacterial culture was put on a clean glass slide one drop of 3% KOH solution. KOH solution was placed over it and thoroughly mixed with the help of needle. Bacterial chromosomes seperated out as a thin threads indicated gram -ve bacteria.

3.6.10 Hydrogen sulphide production test:

Material required

24-48 hrs old slants of bacteria culture
Inoculation needle
Sulphate indole medium
Spirit lamp.

Procedure

Sulphide Indole Medium

Peptone -	30gm
Beets extract -	3 gm
Ferrous ammonium sulphate -	0.2 gm
Sodium thio sulphate -	0.025 gm
Agar -	20gm
Distilled Water -	1 lit
P^H -	7.0

Inoculated the bacteria into sulphide indole medium and incubated the plates at room temp. 28±2°C After 24-48 hrs examined for presence of black colouration along the line of bacterial colony. Black colour indicated the positive test of organism.

3.6.11 Siderophore Production

Evaluation of the isolates with universal chrome – Azurole assay (CAS) helps in detecting the siderophore production by PGPR (*Pseudomonas fluorescens)*. This assay mainly depends on the colour zone i.e. orange zone against dark blue background a positive indication for the presence of siderophore. All the 10 isolates were screened by CAS method (Schwyn and Neilands 1987) for their ability to produce siderophores. Six ml of 10 mM strength HDTMA (heradecyl trimethyl ammonium

bromide) solution was poured in to 100 ml volumetric flask and further diluted up to 50 ml with deionized water. One and half ml of Fe_3 solution (prepare by mixing 1 mM $FeCl^3$, $6H_2O$ + 10 mM HCL) and 7.5 ml 2 mM aqueous CAS solutions were added into the volumetric flask which subsequently turned the colour of the solution to dark blue. This entire content was added to 41.0 ml distilled deionized water so as to make the final volume to 100 ml. King's B medium (KMB) were added which turned the medium colour to blue. The medium was sterilized and poured in the petriplates @ 20 ml per plate. After solidification spot inoculation was done with each bacterial isolates and the plates were incubated at 28 ± 2°C for 24 hr. Four replicated plates were maintained for each isolates. The observations were recorded for the development of orange halo region surrounding the incubated spot.

3.6.12 Phosphate Solubilizing Activity

Phosphate solubilization test was performed by spot inoculation of test organism on Pikovaskaya's medium. The plates were incubated at 28±1°C for 4-5 days formation of clear inhibition zone around the colony was considered positive for phosphate solubilization.

3.6.13 IAA Production

Some bacteria oxidize tryptophan, an essential amino acid by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The bacterium was inoculated in to tryptone broth and the indole produced during the reaction is detected by adding Kovac's reagent (dimethyl aminoblenaldehyde) which produces a cherry red reagent layer as illustrated.

Tryptophan ⟶ Indole + Pyruvic acid +Ammonia

Indole + Kovac's reagent ⟶ Resin dole dye + water
(Cherry red compound)

Materials required

N agar slants of bacterial culture
1% tryptone broth
Wash bottle
Inoculation needle
Kovac's reagent (dimethyl amino benaldehyde)
Sterile petriplates or test tube.

Procedure

Prepare 1% tryptone broth (dissolve 10 gm of peptone in 1 lit of distilled water) sterile this in autoclave at 151 lbs (121°C) for 15 min. Inoculate the broth with bacterial culture and inoculate the tube at room temp for 48 hrs - 72 hrs.

After that add 1ml of kovacs reagent to each and control plate. Allow the tubes for stands for 10-15 min. and observed the tubes for development of Red colour in top layer of test tube. It indicated positive reaction and absence of red colour is negative reaction.

Inferance

Development of real colour in tryptone (broth) of test has shown +ve reaction.

3.6.14 *In vitro* antibiosis

The PGPR *Pseudomonas fluorescens* isolates were further tested for their antagonistic ability against three soil borne plant pathogens i.e. *Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii.*

The bacterial isolates were screened by dual culture test as followed by Morton and Strouble (1955) and Ramnathan *et al.* (2002). In petriplates containing 20 ml PDA medium (without antibiotics) and loopful of fresh bacterial culture was streaked at centre and fungal mycelial disk at sides towards the edge of petriplates (Kumar *et al.* 2002) and petriplates were incubated at 28°C for 5-7 days. The percent inhibition of test fungus with each bacterial isolate was calculated. Three replicated plates were maintained for each isolate. Plates streaked with sterilized water in place of bacterial isolates were kept as control. The percent growth inhibition was calculated using following formula.

$$I = \frac{C - T}{C} \times 100$$

Where

T = per cent inhibition

C = Growth of fungus in control (mm)

T = Growth of fungus in Treatment (mm)

3.7 Effect of PGPR on germination of soybean by towel paper method

Effect of PGPR on the vigour of soybean (JS -335) seedling was noted by paper towel method. Pretreated seeds with PGPR @ (106 cfu/ml) prepared @ 5ml/kg were placed on paper towel. Observations viz. germination percent, shoot length (cm), root length (cm) and seedling vigour index were recorded on 5^{th} day. Seedling vigour index was calculated by following formula.

SVI = Percentage germination x {shoot length (cm) + root length (cm)}

Where

SVI = seedling vigour index.

Statistical analysis

Statistical analysis was done by using methods of analysis of variance and mean were tested for significance. Critical Difference were used for comparison. Whenever the critical difference were found to be significant an indicated by 'F' test. (Panse and Sukhatme, 1967).

Chapter IV

RESULTS AND DISCUSSION

The investigation on "Biochemical characterization of PGPR of soybean (*Glycine max* L. Merill)" was carried out during 2011-2012. The results and its interpretation of present studies are given in this chapter.

Soil borne fungal pathogens including *Fusarium oxysporum* f. sp. ciceri, *Sclerotium rolfsii* and *Rhizoctonia solani* causing wilt of chickpea, root rot of soybean and stem rot of groundnut respectively are the major constraints and causes considerable yield losses in cereal and pulses annually.

Biological control has proven to have great potential in reduction of several soil borne diseases. Amongst them *Pseudomonas fluorescens* is potential growth promoters and antagonist against soil borne pathogens. It dominates in the rhizosphere and possesses several properties that have made them as bicontrol agent of choice.

The mechanisms by which these bacteria affect the plants involve the production of diverse metabolites including siderophore, phytoharmones and the other associated activities which include phosphate solubilization, iron competition in soil and root colonization resulting in plant growth promotion.

Hence native strains of PGPR *P. fluorescens* were isolated from rhizosphere of soybean (*Glycine max*. L. Merill). Observations were recorded on growth parameters. Rhizospheric population, morphological and biochemical test of *P. fluorescens* isolates. Studies were also carried on per cent growth inhibition and antibiosis of *P. fluorescens* isolates against the soil borne fungal pathogens.

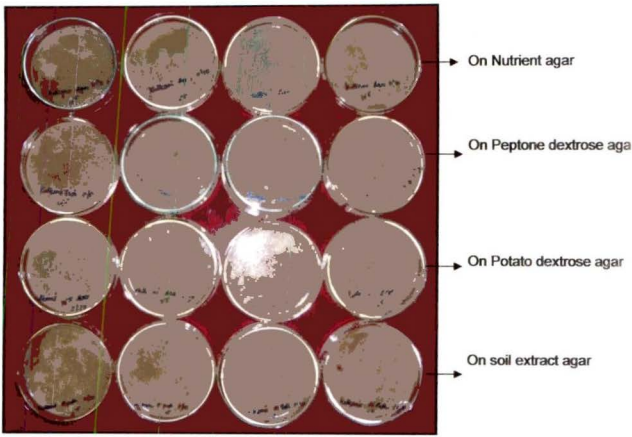
4.1 Collection of soil samples and Isolation of PGPR *Pseudomonas fluorescens, Enterobacter*

Soil samples were collected from rhizosphere of soybean (*Glycine max* L. Merill) from different farms of College of Agriculture, Nagpur and were processed in the laboratory for isolation of *Enterobacter* on Eosin methylene blue agar medium and *P. fluorescens* on King's B medium by serial dilution and pour plate method.

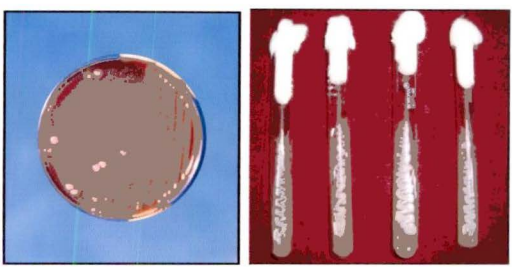
After three days of incubation greenish yellow to whitish yellow colonies were obtained on King's B medium which were later picked streaked on fresh King's B medium for pure culture and used for investigation. These isolates were designated as *Pseudomonas fluorescens* as Pf1, Pf2, Pf3 and Pf4. The isolates of *Enterobacter sp* were designated as A, B, C and D.

Table 1: Isolation of PGPR (*Pseudomonas, Enterobacter)* from various locations

Sr. No	Location	*Pseudomonas fluorescens*	Rhizospheric population (Cfu)x 10^6	*Enterobacter*	Rhizspheric population (Cfu)x 10^6
1	Agronomy research field, COA, Nagpur.	Pf 1	17	A	15.5
2	ACSS Research Field, COA, Nagpur.	Pf 2	15.5	B	12.5
3	Entomology Research Field, COA, Nagpur.	Pf 3	13.5	C	13.2
4	Pathology Research Field, COA, Nagpur.	Pf 4	9.5	D	14.5



Isolation of PGPR on four non selective media.



***Enterobacter* isolate**

Pf1 Pf2 Pf3 Pf4

Isolate of PGPR (*Pseudomonas* isolates)

Plate 1 : Isolates of PGPR (*Pseudomonas, Enterobacter*).

The data presented in Table 1 shows the location wise isolate of *P. fluorescens*, four isolate of *P. fluorescens* were obtained from rhizospheric soil of soybean (*Glycine max* L. Merill.) and were designated as Pf1, Pf2, Pf3 and Pf4.(Plate 1).

Rhizospheric population of *P. fluorescens* varied from 9.5 x 10^6 to 17x10^6. Maximum rhizospheric population was obtained from Pf1 (17x10^6) followed by Pf2 (15.5 x 10^6). Minimum rhizospheric population was of Pf4 (9.5 x 10^6) obtained from soil of Plant Pathology Research farm, College of Agriculture, Nagpur.

Yeon *et al.* (2009), Mahmoud *et al.* (2010), Khakipour *et al.* (2008) and Sarode *et al.* (2009) isolated the *Pseudomonas* from rhizosphere of rice, Iran and wheat respectively.

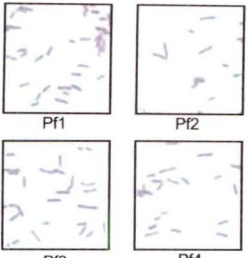
Rhizospheric population of *Enterobacter spp* varied from 12.5 x 10^6 to 15.5x10^6. Maximum rhizospheric population was obtained from A (15.5x10^6) followed by D (14.5 x 10^6). Minimum rhizospheric population was of B (12.5 x 10^6) obtained from soil of Soil Science Research farm, College of Agriculture, Nagpur.

Carlos *et al.* (2011) and Mallesh (2008) isolated the *Enterobacter* from rhizosphere of *Aerucaria angustifolia* and Ashwagandha respectively.

Table 2: Morphological and biochemical tests of *Pseudomonas fluorescens* isolates

Sr. No	Characters	Reaction of Isolates			
		Pf 1	Pf 2	Pf 3	Pf 4
Morphological Properties					
1	Gram Reaction	-ve	-ve	-ve	-ve
2	Cell Shape	Rod	Rod	Rod	Rod
Physical and Biochemical Properties					
3	H_2S Production	+	+	+	+
4	Gelatin Liquefaction	+	+	+	+
5	Starch Hydrolysis	+	+	+	+
6	Pyocynin Test	+	+	+	+
7	Fluorescens Test	+	+	+	+
8	Motility	+	+	+	+
9	Oxidase Test	+	+	+	+
10	KOH Test	+	+	+	+

It can be seen from Table 2 that all isolates were rod and gram negative in reaction. All isolates were found efficient to liquefy gelatin (Plate 2) (Tiwary and Thrimurthy, 2007) and were found capable of H_2S production (Plate 4) (Shinde 2003).Among these all the isolates were able to hydrolyse the starch and showed positive test for starch hydrolysis (Plate 4), Pyocynin, Fluorescens (Plate 2), Motility, Oxidase(Plate4), KOH test. (Plate 4). These findings are in accordance with the characteristics published in Bergey's manual of determinative bacteriology. (Breed *et al.* 1957) and also with the earlier reports (Hegedoron, 2001, Bhatia *et al.* 2005, Siddiqui and Shakeel, 2009).



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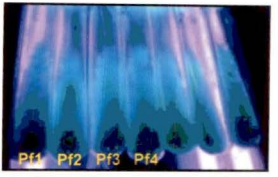
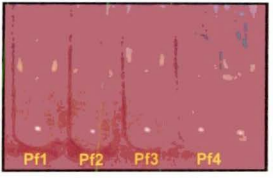
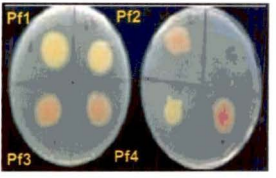


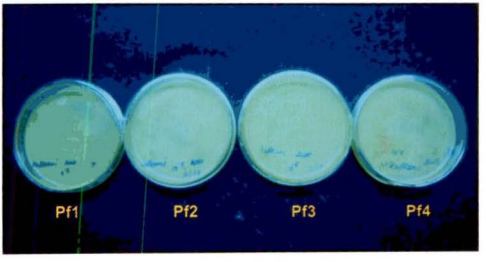
Fluorescence under UV light



Gelatin liquefaction

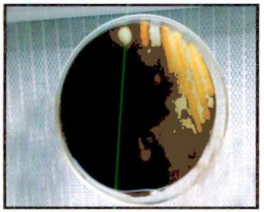


Pyocynin test

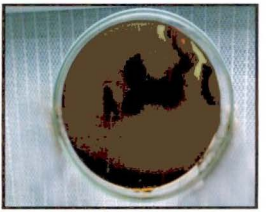


Reaction to UV light

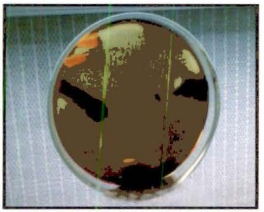
Plate 2 : Biochemical characterization of PGPR.



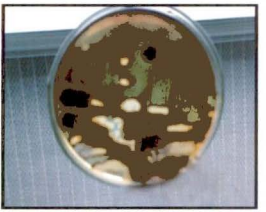
Pf1



Pf2



Pf3



Pf4

Starch hydrolysis

H_2S Production

KOH Test

Plate 4 : Biochemical characterization of PGPR.

The isolates responded to the gram negative and were rods that produced round to irregular colonies and with yellowish to dull yellowish and greenish yellowish water soluble pigment production. This confirmed isolates as *P. fluorescens*. The biochemical tests i.e. gelatin liquefaction, H_2S production, starch hydrolysis, Fluorescens test, Pyocynin test, oxidase test and chrome-azurole S assay for siderophore production further confirmed to be *P. fluorescens* (Qing Ping hu *et al.* 2011, Alka Gupta and Gopal 2008, Aditi Bhattacharya 2010, Krey *et al.* 2005, Yeole and Dube (2000 and 2001). However variable reaction of *Pseudomonas fluorescens* has been reported by Mahesh (2007).

Table 3: Morphological and biochemical tests for PGPR (*Enterobacter)* isolates

Sr. No	Characters	Reaction of Isolates			
		A	B	C	D
Morphological Properties					
1	Gram Reaction	-ve	-ve	-ve	-ve
2	Cell Shape	Rod	Rod	Rod	Rod
Physical and Biochemical Properties					
3	H_2S Production	-	-	-	-
4	Gelatin Liquefaction	-	-	-	-
5	Motility	+	+	+	+
6	Oxidase Test	-	-	-	-
7	Siderophore production	+	+	+	+
8	Phosphate solubilization	-	-	-	-
9	Tryptophan deaminase	-	-	-	-
10	Indole production	-	-	-	-

It can be seen from Table 3 that all the isolates were rod shaped and gram negative in reaction. All the isolates shows positive test for motility test(Plate 3) and Siderophore production (Plate 6) while all the isolates showed negative test for H_2S production, gelatin liquefaction, oxidase test, phosphate solubilization, tryptophan deaminase and Indole production.

Table 4: Growth promotion activities of PGPR (*Pseudomonas fluorescens and Enterobacter spp.)* isolates

Sr. No.	Isolates	Siderophore Production	Phosphate Solubilization Activity	IAA Production	Amount of IAA Produced mg/l
1	Pf 1	+	+	+	28.2
2	Pf 2	+	+	+	17.7
3	Pf 3	+	+	+	25.1
4	Pf 4	+	+	+	21.5
5	A	+	-	-	-
6	B	+	-	-	-
7	C	+	-	-	-
8	D	+	-	-	-

The findings presented in Table 4 indicate growth promoting characteristics of *P. fluorescens* were able to chelate Fe3+ from chrome azurole agar medium. All isolates produced clear orange zone against dark blue background on King's B medium. (Plate 6). Similar observation for siderophore production has been demonstrated earlier Alka Gupta and Murali Gopal (2008), Sayyed *et al.* (2005) and Sayyed *et al.* (2010) indicating the plant growth promoting rhizobacteria (PGPR) produced siderophores which was detected via chrome azurole S assay which is general test for siderophores production.

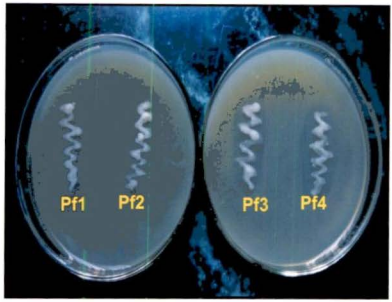


Plate 5 : Phosphate solubilization activity of PGPR isolates.

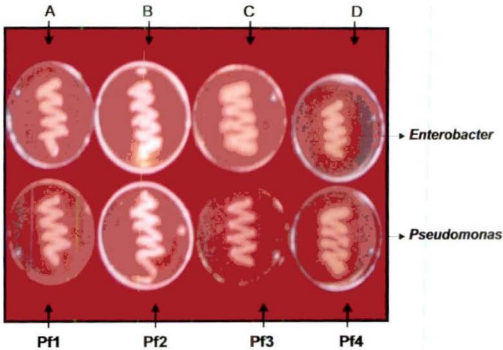


Plate 6 : Siderophore production by PGPR isolates.

Development of pink colour upon addition of Kovac's reagent to culture supernatant of *P. fluorescens* strain confirmed IAA production. All the isolates showed positive test for IAA production. Production of IAA and some other auxins has also been reported in the culture of *P. fluorescens* isolated from rice (*Oryza sativa*) (Mahmoud Reza Ramezanpour *et al.*, 2010). The IAA production in presence of tryptophan (50 gm L^{-1}) in all isolates of *Pseudomonas* i.e. Pf1, Pf2, Pf3 and Pf4 was recorded within the range of 17.7 – 28.2 mg/l. These observations are in line with reports of Shinde (2003), Maleki *et al.* (2010), Husen (2003) and Bhromsiri and Bhromsiri (2010).

However there was no IAA production in the isolates of *Enterobacter* (A, B, C & D) indicating that they do not possess the ability to produce IAA. Muzumdar *et al.* (2007) showed soluble ability of *Pseudomonas fluorescens*. Siddiqui and Shakeel (2009) characterized the isolates of *Pseudomonas fluorescens* on biochemical reaction.

Phosphate solubilization by bacterial strains was found positive as they formed clear zone on pikovaskayas agar medium. All the four isolates produced clear zone (plate 5). Similarly two strains of phosphate solubilizing bacteria from soil sample identified as *P. fluorescens* and *Bacillus megaterium* on the basis of their morphological, cultural and biochemical reactions. Their phosphate solubilizing efficiency confirmed on pikovaskayas medium. (Sharma *et al.* 2007). Siddiqui and Shakeel (2009) also observed clear zone produced by *Pseudomonas* on Pikovaskaya's medium by all twenty one isolates. Positive test of *Pseudomonas* in respect of oxidase, mobility have been documented by Nathan *et al.* (2011).

On nutrient agar medium all isolates showed slow growth and produced dull yellowish coloured colonies. All the isolates produced round shaped colonies except Pf2 where they produced irregular colonies. Irregular colonies produced because of composition of media.

It is observed from this study that growth of *P. fluorescens* was fast on King's B medium as compared to other medium. Most of the *P. fluorescens* produced greenish yellow coloured colonies on King's B medium.

4.2 Effect of PGPR on growth of *Fusarium oxysporum* f. sp. ciceri, *Sclerotium rolfsii and Rhizoctonia solani* at different duration *in vitro*.

Observations on average colony diameter at 3, 5 and 7th DAI and per cent growth inhibition was recorded. All isolates under the test showed their potentiality to check the mycelial growth of all three pathogens i.e. *Fusarium oxysporum*, *Sclerotium rolfsii* and *Rhizoctonia solani*.

Table 5: Antifungal activity of PGPR isolates against *Rhizoctonia solani*

Sr. No	PGPR Isolates	Mycelial Growth (mm) DAI			Growth Inhibition (%) DAI		
		3	5	7	3	5	7
1	Pf 1	40.30	61.40	74.90	31.11	28.60	16.77
2	Pf 2	39.60	64.60	68.40	32.30	24.88	24.00
3	Pf 3	39.90	61.90	70.60	31.79	28.02	21.55
4	Pf 4	41.00	62.00	69.60	29.91	27.90	23.33
5	Control	58.50	86	90.00	-	-	-
	F Test	Sig	Sig	Sig			
	SE(m)±	0.268	0.361	1.086			
	CD (P=0.01)	1.079	1.456	4.372			

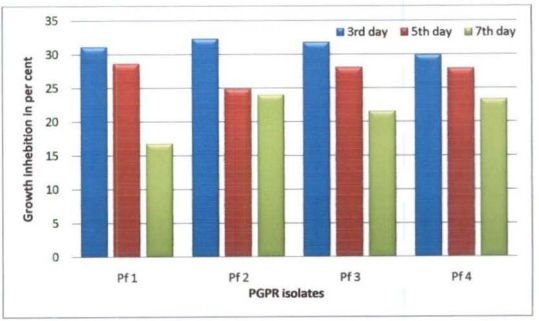


Figure 1: Antifungal activity of PGPR isolates against *Rhizoctonia solani*.

The data presented in Table 5 Indicates that there were significant differences in radial mycelial growth due to various isolates over uninoculated control. Minimum radial mycelial growth was recorded by the isolate Pf2 (68.40 mm) and it was at par with all the isolates followed by Pf4 (69.60 mm) with per cent inhibition of 24.00 and 23.33% on 7th DAI respectively. (Fig. 1 and Plate 7). All the four isolates produced antifungal compounds which have inhibited the growth of *R. solani*. The rusult under present study are in accordance with earlier reports by Paolina Garbeva *et al.* (2004), Nandkumar *et al.* (2002), Sindhu and Dadarwal (2001), Siddiqui *et al.* (2002), Nagrajkumar *et al.* (2004) and Battu and Reddy (2009) showed the antifungal activity of *Pseudomonas* against *R. solani*.

Table 6: Antifungal activity of PGPR isolates against *Sclerotium rolfsii*

Sr. No	PGPR Isolates	Mycelial Growth (mm) DAI			Growth Inhibition (%) DAI		
		3	5	7	3	5	7
1	Pf 1	22.80	42.20	66.60	27.61	25.30	26.00
2	Pf 2	23.10	46.00	66.90	26.66	18.58	25.66
3	Pf 3	22.30	45.50	65.10	29.20	19.46	27.66
4	Pf 4	25.20	45.90	66.70	20.00	18.76	25.88
5	Control	31.50	56.50	90	-	-	-
	F Test	Sig	Sig	Sig			
	SE(m)±	0.301	0.223	0.589			
	CD (P=0.01)	1.213	0.899	2.373			

Similarly the antifungal activity of PGPR was tested against *Sclerotium rolfsii* and the data is presented in Table 6 and depicted in fig. 2 and Plate 7.

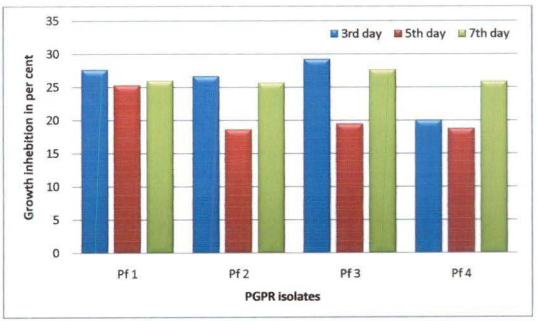


Figure 2: Antifungal activity of PGPR isolates against *Sclerotium rolfsii*.

The observation were recorded at various interval i.e.3, 5 and 7th DAI. It was revealed from the data that significant differences at all the intervals. Minimum radial mycelial growth was recorded by the isolate Pf3 (65.10 mm) and it was followed by Pf1 (66.60 mm) with maximum per cent inhibition 27.66% and 26% respectively. All the isolates were at par with each other.

Pf2 showed less per cent growth inhibition (25.66 %) at 7th DAI. These observations are in agreement with the findings of earlier workers Manjula *et al.* 2004, Bhatia *et al* 2005. *P. fluorescens* was identified as bicontrol agents of ground nut stem rot and other soil borne disease. (Baig *et al.* 2002, Gholami *et al.* 2009, Saharan and Nehra 2011)

Table 7: Antifungal activity of PGPR isolates against *Fusarium oxysporum* f. sp. ciceri

Sr. No	PGPR Isolates	Mycelial Growth (mm) DAI			Growth Inhibition (%) DAI		
		3	5	7	3	5	7
1	Pf 1	25.30	45.70	63.30	25.58	15.37	29.66
2	Pf 2	24.60	47.50	64.90	27.64	12.03	27.88
3	Pf 3	25.60	46.40	64.70	24.70	14.07	28.11
4	Pf 4	24.30	47.30	67.60	28.52	12.40	24.88
5	Control	34	54	90	-	-	-
	F Test	Sig	Sig	Sig			
	SE(m)±	0.286	0.176	0.324			
	CD (P=0.01)	1.166	0.708	1.303			

A termed of data in Table 7 indicates significant differences at 3, 5 and 7th DAI on radial mycelial growth of *F. oxysporum* f.sp. ciceri due

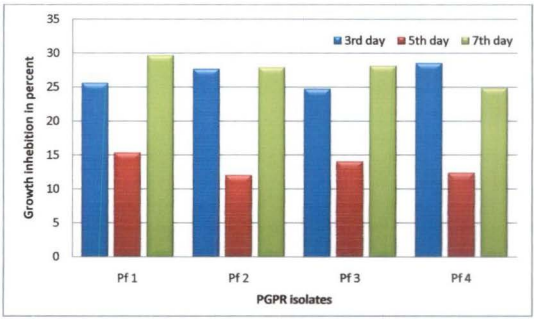


Figure 3: Antifungal activity of PGPR isolates against *Fusarium oxysporum* f.Sp. ciceri

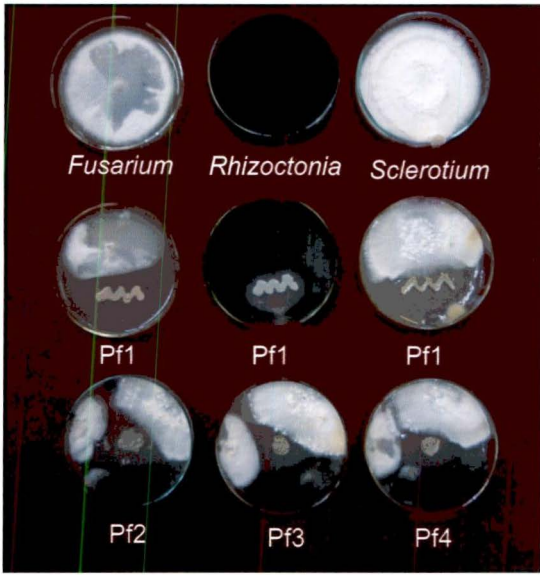


Plate 7: **Antagonism activity of PGPR isolates against *Fusariom oxysprum*, *Sclerotium rolfsil*, *Rhizoctonia solani*.**

to PGPR isolates. Minimum radial mycelial growth was recorded by Pf1 isolates (63.30mm) followed by Pf 3 isolate (64.70 mm) with maximum per cent inhibition 29.66 and 28.11% respectively. (Fig. 3 Plate 7). and This may be due to the antibiotic production of PGPR. The observation recorded in the present investigation are in supported the findings of Rajeshwari and Kannnabiran (2011), Salam *et al.* (2007), Kumar *et al.* (2005) Saravanan *et al.* (2004), Ramamoorthy *et al.* (2002) and Jayswal *et al.* (1990) reported the antagonistic activity of *Pseudomonas* strain against *Fusarium monoliformae*.

The results shows that the plant growth promoting rhizobacteria *Pseudomonas fluorescens* can play an important role in bicontrol of soil borne diseases of rhizosphere.

Table 8: Effect of PGPR isolates on germination percentage and vigour index.

Sr . No.	PGPR Isolate	Germination Percentage	Mean Shoot Length (cm)	Mean Root Length (cm)	Vigour Index
1	Pf 1	77	9.27	15.11	1878.21
2	Pf 2	73	8.31	14.55	1646.28
3	Pf 3	74.66	9.13	14.21	1744.52
4	Pf 4	74.33	8.53	14.38	1704.40
5	Control	72.33	7.47	9.25	1275.74

The result with regard to effect of PGPR on germination percentage, shoot length, root length and vigour index are presented in Table 8 and depicted in fig 4, 5, 6 and 7 respectively and plate 8. Seed treatment with PGPR isolates Pf 1 exhibited higher germination i.e. 77% after 7 days respectively when tested by towel paper method. The seedling vigour index was maximum in PGPR isolate Pf1 1878.21 followed by Pf 3 1744.52. Maximum shoot length was observed in Pf1

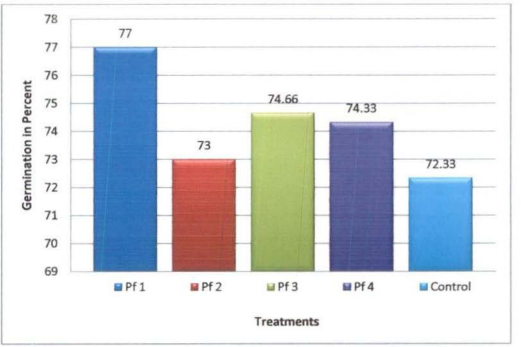


Figure 4: Germination percentage induced by PGPR isolates in Soybean.

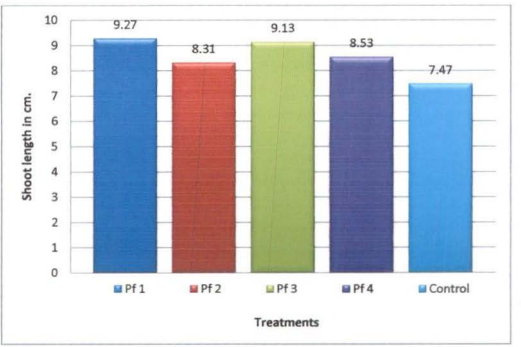


Figure 5: Effect of PGPR isolates on shoot length.

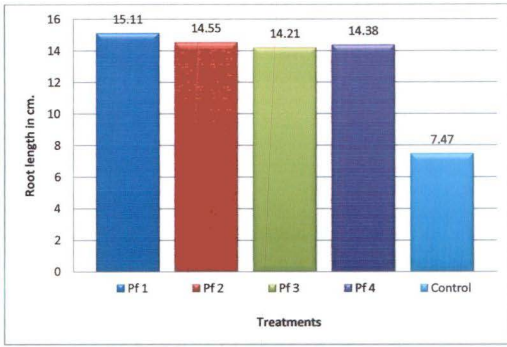


Figure 6: Effect of PGPR on root length.

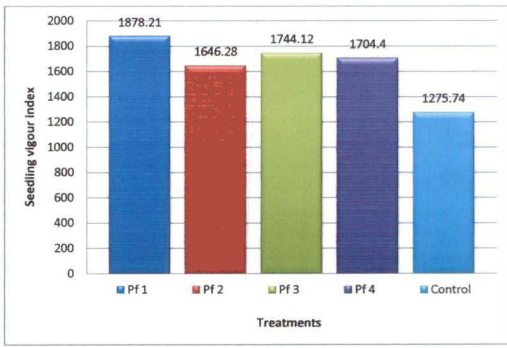
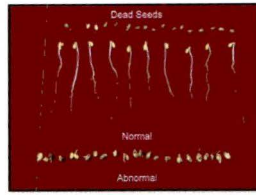
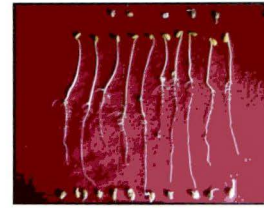


Figure 7: Seedling Vigour index influenced by the different PGPR isolates.

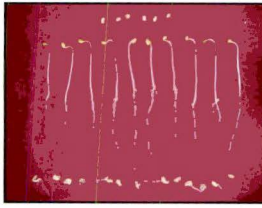


Control

Isolate 1



Isolate 2



Isolate 3

Isolate 4

Plate 8: Effect of PGPR isolates on germination of soybean seeds.

i.e. 9.27 cm while maximum root length was in Pf1 (15.11 cm) as compared to control. Minimum shoot length was observed in control followed by Pf 2 (8.31 cm) and root length was in Pf 3 (14.21 cm). It indicates that PGPR isolates were effective for soybean as seed dresser for higher seedling vigour index and it does not have detrimental effect to other useful bioagents.

PGPR isolates were inoculated to soybean seeds and seedling vigour was recorded. The beneficial effects of rhizosphere micro-organisms have been studied for several crop species. Garima Jha *et al.* (2005), Carlos Marcalo Riberio *et al.* (2011), Hynes *et al.* (2008), Naz *et al.* (2009),Chaiharn *et al.*(2008). The plant growth promoting activity of rhizobacteria such as *Azotobacter, Azospirillum, clostridium, Pseudomonas fluorescens* are well established (Kloepper *et al.* 1980). PGPR promote plant growth or inhibit the soil borne pathogen include the production of extra cellular growth promoting chemical substances and from chelating siderophores. Seed treatment with PGPR, Pf 1 exhibited higher germination in soybean i.e. 77% respectively as compared to control. 72% when tested by towel paper method. Similar results were confirmed the findings of reported that the treatment of rhizobacteria with soybean seed increased the germination of soybean. The seedling vigour index was maximum i.e. 1878.21 in PGPR Pf1 followed by 1744.52 in PGPR Pf3 root length and shoot length was also maximum in respective isolates. The present findings are on results published by Sakthivel *et al.* (1986), Sayyed *et al.* (2005) and Begum *et al.* (2003). Their results suggest the possible use of PGPR as seed dresser in Soybean for seed germination seed protection from seed borne and soil borne pathogen, plant growth promotion. Further testing is needed with recent biotechnological tools with large no. of PGPR isolates from wide geographical areas.

Chapter V

SUMMARY AND CONCLUSION

The interaction of microbe with the plant in the rhizosphere can be beneficial, neutral, antagonist or deleterian for plant growth. The plant growth promoting rhizobacteria (PGPR) colonises the root aggressively in the rhizosphere and termed as rhizobacteria. The present investigation entitled, "Biochemical characterization of PGPR of soybean (*Glycine max* (L) Merill)" was carried out during the year 2011-12 at Plant Pathology Section, College of Agriculture, Nagpur.

Four soil samples from different location in the vicinity of College of Agriculture were collected from rhizosphere region of soybean plants. These samples were processed for isolation of PGPR on different media. These rhizobacteria were designated as Pf1, Pf2, Pf3 and Pf4 which shows the characteristics of *Pseudomonas*. Morphological and Biochemical characteristics were studied for gram reaction, cell shape, H_2S production, gelatin liquefaction, starch hydrolysis, pyocynin test, motility, oxidase and KOH test. All the isolates were gram – ve, rod shape, and all the showing the positive tests as above.

Similarly their root rhizosphere soil samples were also studied for isolation of *Enterobacter* using Eosin methylene blue agar medium. Four isolates belonging to genera *Enterobacter* were entitled and designated as A, B, C and D for further studies. Morphological characters were studied, all the isolate were gram – ve and rod shaped. These isolates did not produce H_2S and –ve in gelatin liquefaction test. However motility and siderophore production were positive but phosphate solubilization, tryptophan deaminase, and IAA production were negative which synonym to the characteristics of *Enterobacter*.

With regard to phosphate solubilization activity and IAA production of *Pseudomonas* isolates i.e. Pf1, Pf2, Pf3 and Pf4 exhibited phosphate solubilizing activity and produced IAA ranging from 17.7 to 28.2 mg/L. However it was negative in *Enterobacter* isolate.

Antagonistic activity of *Pseudomonas* isolates were tested against *Fusarium oxysporum* f.sp. ciceri, *Rhizoctonia solani* and *Sclerotium rolfsii*. It was revealed from data, the significant differentiation, radial mycelial growth at 3, 5 and 7^{th} DAI over uninoculated control. Minimum radial mycelial growth was noticed in Pf2 isolate with maximum per cent inhibition in *R. solani*, Pf3 isolate in *S. rolfsii* and Pf1 in the *F. oxysporum* f.sp. ciceri.

The results in respects of germination percentage, shoot, root length and vigour index reveals that the isolate Pf1 was found better in all above respect followed by Pf3.

It can be concluded from the present investigation that the PGPR isolate (*Pseudomonas spp*) can be used for controlling soil borne plant pathogens. However there studies are *in vitro* and one year experimentation therefore needs further confirmation.

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Manoj

Signature of Student

APPENDIX

1. King's B medium:

Proteose peptone no. 3	-	20 gm
Glycerol	-	10 ml
Asparagine	-	2.25 gm
K_2HPO_4	-	1.5 gm
$MgSO_47H_2O$	-	1.5 gm
Agar	-	15 gm
Distilled water	-	1 L
Cyclohaxamide	-	1 mg

2. Glucose Peptone Agar:

Glucose	-	5.0 gm
Peptone	-	10 gm
Bromo cresol purple	-	10 ml
Agar	-	15 gm
Distilled water	-	1 L
Cyclohexamide	-	1mg

3. Nutrient Agar :

Beef extract	-	3 gm
Peptone	-	5 gm
Agar	-	15 gm
Distilled water	-	1 L
Cyclohexamide	-	1 mg

4. Potato Dextrose Agar:

Peeld potato	-	200 gm
Dextrose	-	20 gm
Agar	-	15 gm
Distilled water	-	1 L
Cyclohexamide	-	1 mg

5. Soil Extract Agar:

Glucose	-	1.0 gm
K_2HPO_4	-	0.5 gm
Agar	-	15 gm
Soil extract	-	100 ml
Tap water	-	900 ml
Cyclohexamide	-	1 mg

6. Eosin Methylene Blue Agar:

Peptone	-	10 gm
Lactose	-	10 gm
K_2HPO_4	-	2 gm
Agar	-	15 gm
Eosin Y	-	0.4 gm
Methylene Blue	-	0.065 gm
Distilled water	-	1 L
Cyclohexamide	-	1 mg