CHAPTER 2

2.0 ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIAL STRAINS OF FLUORESCENT PSEUDOMONADS

2.1 INTRODUCTION

2.1.1 Development of biocontrol strains of fluorescent *Pseudomonad*:

General strategy to develop effective biocontrol strains involves: collection of diverse isolates from soils naturally suppressive to phytopthogen, screening for biocontrol activity in laboratory and green house conditions, identify biocontrol mechanism, collect new strains preferably from the type of soils and agroclimatic conditions of the region where it is to be applied with similar conserved biocontrol mechanism and utilization of new isolated strain to expand biocontrol programme (Fig. 2.1).

Motile rhizobacteria colonize the rhizoid zones of plant more profusely than non-motile organisms resulting in better rhizosphere activity (Weller, 1988). In the rhizosphere root exudates are the primary source of nutrients and a good root colonizer is well adapted to the utilization of nutrients (Van Overbeek, 1995). An effective PGPR strain should survive well in the rhizosphere and utilize nutrients exuded by the plant roots, proliferate, able to efficiently colonize the entire root system and be able to compete with the endogenous bacteria (Bloemberg, 2001).Certain species of fluorescent pseudomonad e.g. *Pseudomonas chloroaphis, P. fluorescens, P. veronii, P. putida* have received attention because they possess abilities to influence plant growth and development through different mechanism (Weller, 1988; O'Sullivan and O'Gara, 1992). They are recognized as being antagonistic to several opportunistic soil-borne fungi (Weller, 1988; Keel et al., 1992) and to seed borne fungi (Hokeberg et al., 1997). Also, some strains are responsible for significant plant promoting effects (DeFreitas and Germida, 1991; Kropp et al., 1996). Taxonomically, fluorescent *Pseudomonas* spp. belongs to the rRNA group I of the gamma subclass of Proteobacteria (Palleroni, 1993; Kersters et al., 1996). Fig. 2.2 depicts the different groups of *Pseudomonas* and the position of *P. fluorescens* in the phylogenetic tree (Mulet et al., 2010).

2.1.2 Standard plant growth promoting strains of fluorescent *Pseudomonas*:

2.1.2.1 *Pseudomonas fluorescens* CHA0:

P. fluorescens CHA0 (*Pf* CHA0) is a model biocontrol strain for which the importance of antimicrobial metabolites in disease suppression has been demonstrated in several crop-pathogen

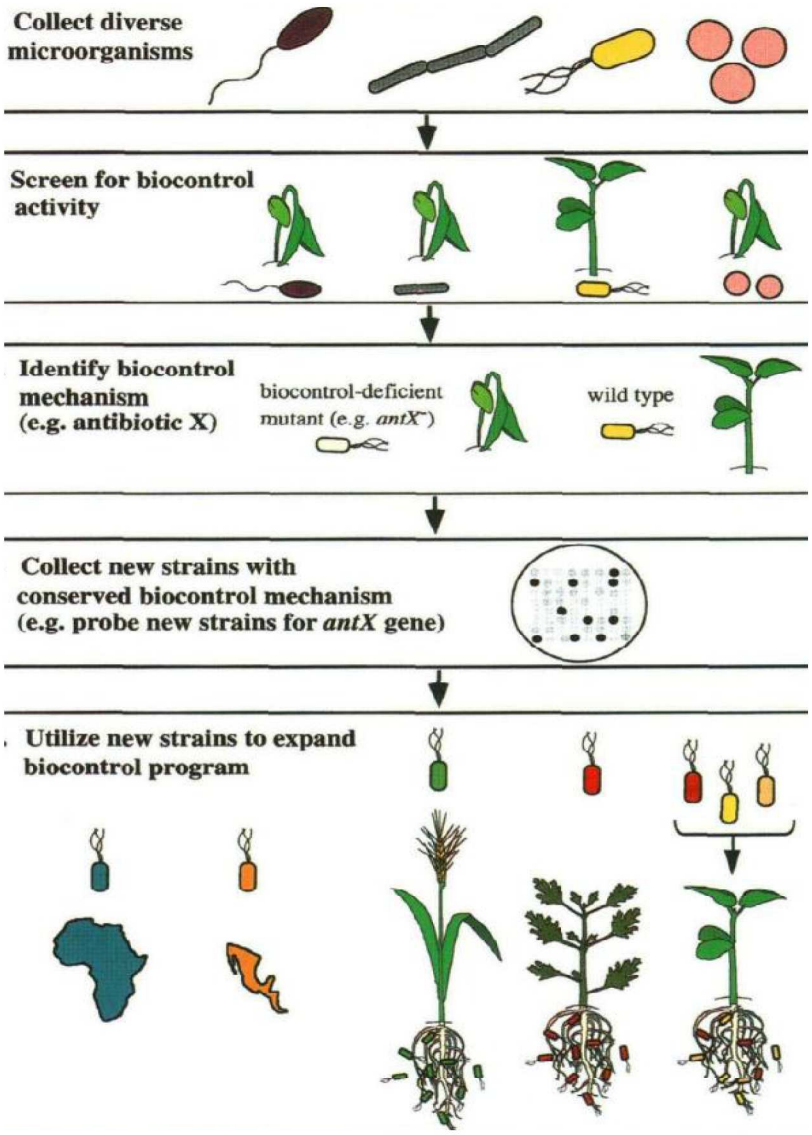


Fig. 2.1: General Strategy used for the isolation of biocontrol strains (González et al., 2010)

Systems and the genetics of antibiotic and siderophore biosynthesis have been well characterized. (Voisard at al.,, 1994). *Pf CHA0* was isolated from a Swiss soil naturally suppressive to black root rot of tobacco caused by *Chalara elegans* (synanamorph *Thielaviopsis basicola*). *Pf* CHA0

reduces the extent of disease caused by several root-pathogenic fungi such as *Thielaviopsis basicola*,

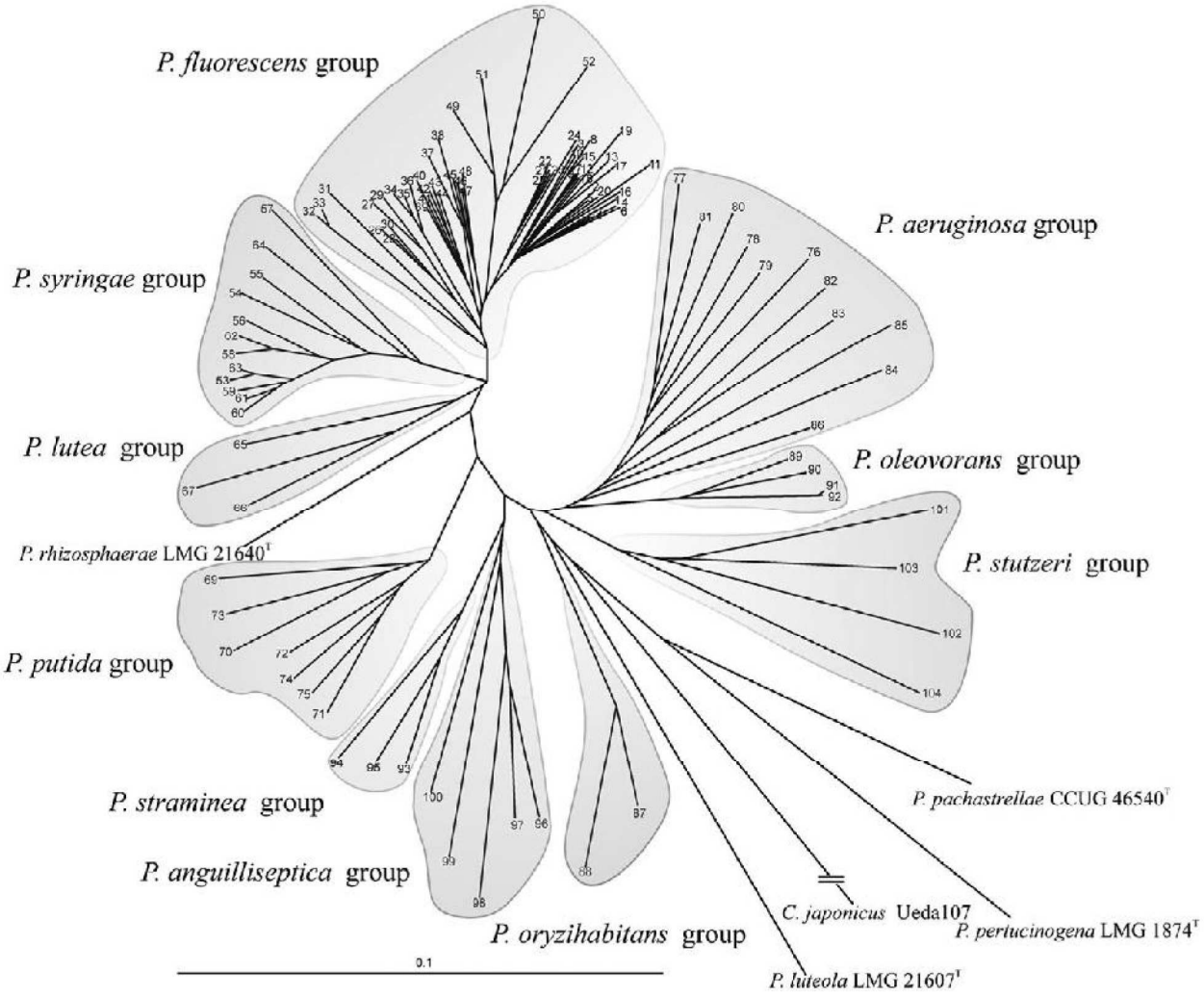


Fig. 2.2: Phylogenetic tree of *Pseudomonas* spp. (Mulet et al., 2010)

Gaeumannomyces graminis var. *tritici* (Ggt), *Pythium ultimum*, *Rhizoctonia solani*, and *Fusarium oxysporum* (Defago et al., 1990). In many of these studies, production of 2, 4 – Diacetyl phluoroglucinol (2, 4-DAPG) has emerged to be a key factor in the biological control activity of *Pf* CHA0 (Keel et al., 1992, 2007). In addition to 2,4 - DAPG, the strain produces the HCN (Voisard et al., 1989), antimicrobial metabolites pyoluteorin (Defago et al., 1990), the siderophores pyoverdine, pyochelin (Voisard et al.,, 1994) and salicylic acid (Meyer et al., 1992), the phytohormone indole-3-acetic-acid. *Pf* CHA0 has been a model organism to identify biosynthetic genes of HCN and 2, 4 - DAPG and study regulation thereof (Laville et al., 1992;

Blumer et al., 1999; Heeb et al., 2002). *Pf* CHA0 has been recently redesignated as the type strain of a new species *P. protegens* based on polyphasisc studies using molecular and biochemical/phenotypic data (Ramette et al., 2011). However, in this work it is referred to as *Pf*CHA0.

2.1.2.2 *Pseudomonas fluorescens* Pf-5:

P. fluorescens Pf-5 is a rhizosphere bacterium that suppresses seedling emergence diseases and produces a spectrum of antibiotics toxic to plant-pathogenic fungi and oomycetes. The complete sequence of the 7.07 Mb genome of the biological control agent, *P. fluorescens* Pf-5 is available, providing an opportunity to advance knowledge of biological control through genomics (Loper and Gross, 2007). In addition to six known secondary metabolites produced by Pf-5, three novel secondary metabolite biosynthesis gene clusters identified in the genome could also contribute to biological control. The genomic sequence provides numerous clues as to mechanisms used by the bacterium to survive in the spermosphere and rhizosphere. These features include broad catabolic and transport capabilities for utilizing seed and root exudates, an expanded collection of efflux systems for defense against environmental stress and microbial competition, and the presence of 45 outer membrane receptors that should allow for the uptake of iron from a wide array of siderophores produced by soil microorganisms (Loper et al., 2007). As expected for a bacterium with a large genome that lives in a rapidly changing environment, Pf-5 has an extensive collection of regulatory genes, only some of which have been characterized for their roles in regulation of secondary metabolite production or biological control.

2.1.2.3 2, 4- DAPG producers:

The ability of nine crops (alfalfa, barley, bean, flax, lentil, lupine, oat, pea and wheat) to support indigenous populations of DAPG-producers was investigated (de la Fuente et al., 2006). DAPG-producers survive in bulk soil at densities below the detection level, but rapidly proliferate in the rhizosphere. Rhizosphere population densities differed among the various crops and cultivars, with lentil and oat supporting the highest and lowest densities of DAPG-producers, respectively.

In this chapter, the study was focused on the following aspects:

1. Isolation of fluorescent pseudomonad from the rhizosphere of different monocots and dicots from different locations in Gujarat, western India
2. Identification of the bacterial isolates by biochemical and molecular methods

3. Characterization of isolated strains for plant growth promoting traits viz. P-solubilization, antifungal activity, antibacterial assay, siderophore production, IAA production, HCN production, ACC deaminase activity

2.2 MATERIALS AND METHODS:

2.2.1 Rhizospheric soil collection:

Rhizospheric soil samples were collected from various dicot plants e.g. cotton, pigeon pea, tobacco, ground nut and monocots e.g. banana, rice , brinjal, sugarcane from different regions of the agricultural belts of Gujarat like Anand, Ahmadabad, Junagadh, Vadodara and Halol (Panch Mahal) (Fig.2.3) in April and August months of year 2006. Plants were selected from agriculture fields showing good, healthy plant growth. Plants were carefully uprooted from the soil so that the roots and the attached soil were removed intact. Thereafter, roots with the adherant soil were transferred to sterile sample collection bags and packed for transport to the lab. Root samples were transferred to 4°C as soon as possible.

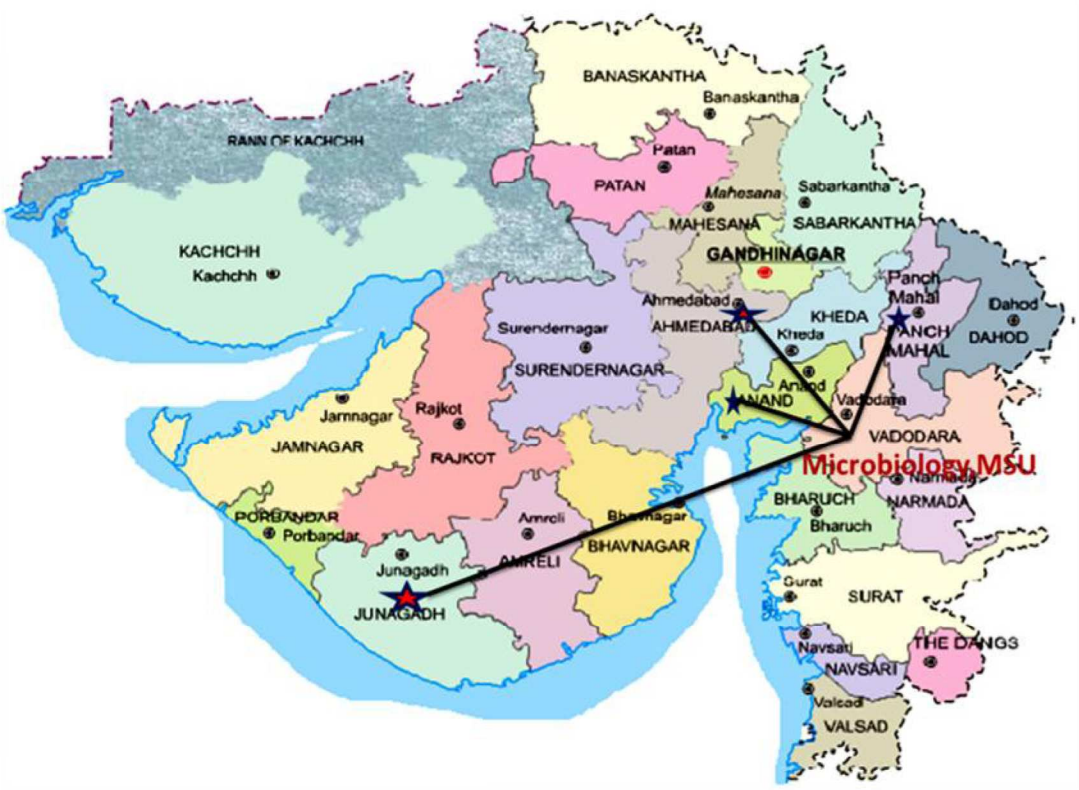


Fig.2.3 Location of sample collection sites on Gujarat map

2.2.2 Isolation of fluorescent pseudomonad strains:

The suspensions of rhizospheric soil and washed roots were prepared in sterile 0.85% NaCl (N-saline) provided that root/adherent soil samples were vigorously vortexes in 20 ml saline. Dilutions was spreaded on King's B plates (KMB) (Hi-Media Company, Mumbai, India), amended with antibiotics cycloheximide (100 µg/ml), chloramphenicol (13µg/ml) and ampicillin (40 µg/ml) (Mcspadden-Gardener et al., 2001). The plates were incubated overnight at 30^0C for 24 h, after which they were observed for the presence of fluorescent green colonies under UV light. Initially isolated fluorescent colonies were not obtained but the patches of microbial growth showing fluorescence were scraped off the plate and resuspended in 1.5 ml of sterile N-saline and vortexed to obtain uniformly distributed suspension of fluorescent cultures. This suspension was used to inoculate the germinated sterile seedlings of Mung bean.

King's B Medium:

Ingredients	(g/L)
Proteose peptone	20.0
KH_2PO_4	1.5
$MgSO_4.7H_2O$	1.5
Glycinc	4.4
Glycerol	15
Distilled Water	1000 ml

Autoclaved at 10 psi for 20 min.

2.2.3 Enrichment method for the isolation of efficient root colonizers fluorescent pseudomonad:

2.2.3.1 Surface sterilization and germination of *Vigna radiata* seeds:

Vigna radiata (Mung bean K831) seeds obtained from Anand Agriculture University, Anand Gujarat, were thoroughly washed 3-4 times with sterile distilled water. Thereafter, seeds were treated with 0.1% $HgCl_2$ for 2 min followed by 2 min treatment with 70% ethanol with vigorous shaking in between. Seeds were washed with sterile distilled water two times to remove traces of $HgCl_2$ and transferred to sterile petriplates containing wet filter paper aseptically and incubated in dark. Sterile distilled water was added on to the filter paper the second day to maintain humidity for proper germination. Seeds were germinated up to the radical size reached to 1 cm.

2.2.3.2 Plant assay for the effective root colonizer

The germinated sterile seedlings were incubated in this suspension of fluorescent bacteria for 45 min and seedlings were allowed to grow in 1/5 diluted Murashige and Skoog (MS) medium containing 1% agar in sugar tubes for 7 d at 30^{o}C in natural dark and light period. The plantlets were monitored for their shoot and root lengths and number of lateral roots. The plants which have shown the good effect on the growth were selected and the roots were given two washes of sterile N-saline so that only those bacteria that have colonized efficiently remained and others get washed off. About 1cm of root tips were cut from main and lateral roots and suspended in 1.5 ml of sterile N-saline, vortexed vigorously for 30 min and 10-5, 10-6 and 10-7 dilutions were plated on KMB agar, incubated for 24 h at 30^{o}C. Appearance of fluorescent colonies was monitored and suspensions of these colonies were made in saline. The bacterial suspensions were again used to inoculate sterile germinated seedlings in the same manner as above. The inoculated plantlets were then used for isolation or efficient root colonizers and total of three cycles of plant inoculations were carried out. At the end of three cycles, bacteria which showed best plant growth were selected and were purified by repeated streaking on KMB plates (Fig. 2.4).

Murashige and Skoog medium macro elements (Hi Media Ltd., India)

Ingrédicnts	(g/l)
Carbon source	0.00
$CaCl_2$ .2 H_2O	0.44
KH_2PO_4	0.17
KNO_3	0.19
$MgSO_4$.7 H_2O	0.37
NH_4NO_3	1.6

Linsmaier and Skoog microelements (Hi Media Ltd., India)

Ingredients	(mg/l)
$CoCl_2$. 6 H_2O	0.025
$CuSO_4$. 6 H_2O	0.025
Na EDTA	37.30
$FeSO_4$. 7 H_2O	27.80
H_3BO_4	6.20
KI	0.83

$MnSO_4.H_2O$	16.90
$NaMo_4.2H_2O$	0.28
$ZnSO_4.7H_2O$	8.60

Pure isolates were routinely grown on KMB plates. Isolates were maintained for long term in 40% glycerol at -20°C.

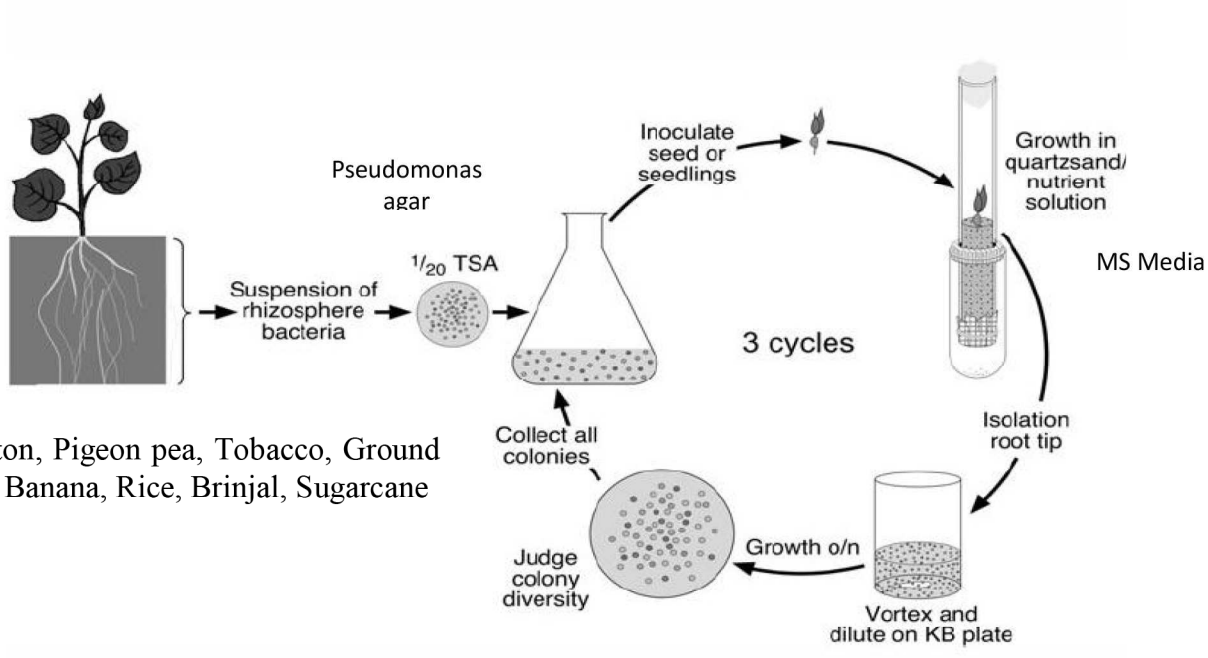


Fig. 2.4 Enrichment technique for the isolation of efficient root colonizing fluorescent pseudomonad

2.2.3.3 Quantification of Antifungal activity:

The dual culture plate test based screening for antifungal activity against the plant-pathogenic fungus *Rhizoctonia bataticola* was performed by placing an agar plug with in the center of a Potato Dextrose Agar (PDA) plate and spotting the *Pseudomonas* isolates on the plate at four locations, 2 cm inside of the plate periphery. Similarly antibiotic extracts were checked by pouring 75 µl of the extracts in wells bored using sterile cork borer at four places at plate periphery. The plates were incubated at 30°C and checked for zones of inhibition of mycelia growth after approximately 3-5 d, when the fungal mycelium reached the edge of the plate. All antifungal tests were performed twice, with new co inoculations used each time. The composition of PDA is as follows (Hi – Media Ltd., India).

PDA Medium:

Ingredients	(g / l)
Potato infusion	200
Dextrose	20
Agar	30
pH	5.1 ± 0.2

The medium was autoclaved at 15 Psi for 15 min.

The extent of fungal inhibition by rhizospheric isolates was assessed by measuring the zone of inhibition of the mycelia radial growth in the plate assay. Inhibition was measured in terms of the distance traveled by the fungus towards the culture and was compared to *Pf* CHA0 on the PDA plate.

2.2.3.4 Quantification of phosphate solubilization ability:

Phosphate solubilizing capability of isolates was checked on Pikovaskya's agar medium (Hi – Media Ltd., India). It contains insoluble tricalcium phosphate, which on solubilization gives rise to clear zones around colonies. Isolates were spotted and incubated at 30^{o}C for 48-72 hrs and were observed for zone of clearance around colonies.

Pikovaskya's agar (Tricalcium – Phosphate Agar):

Ingredients	(g / 100ml)
Yeast extract	0.5
Dextrose	10
$Ca_3(PO_4)_2$	5
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4.7H_2O$	0.1
$MnSO_4$	0.0001
$FeSO_4$	0.0001
Agar	25
D/W	1000 ml

The medium was autoclaved 15 Psi for 15 min.

2.2.4 Identification of fluorescent isolates by biochemical methods:

Bergey's Manual of Determinative Bacteriology, 1994 was referred and the various tests were performed to identify organisms as fluorescent *Pseudomonas* spp. For the tests specifically to be used for fluorescent *Pseudomonas* spp., the protocols from Mac Faddin, 2000 was referred.

2.2.4.1 Gram staining: Smears of the isolated cultures were prepared on grease free slides and stained with Gram staining kit. Fluorescent *Pseudomonas* stain pink as they are Gram negative.

2.2.4.2 Catalase test:

Hydrogen peroxide is formed as an oxidative end product of aerobic breakdown of sugars. Hydrogen peroxide if allowed to accumulate is toxic to bacteria, resulting in their death. Catalase is an enzyme which breaks down H_2O_2 to liberate O_2.

$$H_2O_2 \longrightarrow H_2O + ½\ O_{2\ (g)}$$

Cultures were inoculated in 3ml LB broth and allowed to grow overnight. Few drops of H_2O_2 were added and effervescence indicated presence of catalase. Fluorescent *Pseudomonas* spp. give the test positive being obligate aerobes.

2.2.4.3 Oxidase Test:

The oxidase reaction is due to the presence of a cytochrome oxidase system, which activates the oxidation of reduced cytochrome by molecular oxygen, which in turn acts as an electron acceptor in the electron transfer system. Reduced tetra methyl–p–pheylenediamine dihydrochloride is converted to oxidized tetra methyl–p-phenylenediamine dihydrochloride by oxidase, which is observed as colour change from colorless to deep blue/ purple. Isolates were grown on LB agar. Oxidase discs (Hi-Media Ltd, India) were used wherein the cultures were placed on the disc using sterile tooth pick and then a drop of water was added. The discs were observed for color change from white to blue. Fluorescent *Pseudomonas* spp. gives this test positive being obligate aerobes.

2.2.4.4 Hugh-Leifson's Oxidation Fermentation (OF) test:

Pseudomonad is highly aerobic organisms that grows using oxidation mode. Organisms can utilize carbohydrates either aerobically or anaerobically and some metabolize using both the methods. Presence of oxidation mode and absence of fermentation mode was checked using OF Hugh & Leifson basal medium. Under anaerobic condition organism enters fermentation mode and produces acid which can be checked using bromothymol blue dye, which is green under neutral condition, and yellow under acidic condition. While in aerobic conditions organism

undergoes oxidative mode and produce organic acid which convert green color of bromothymol blue to yellow.

Hugh-Leifson's Oxidation – Fermentation basal medium:

Ingredients	(g/l)
Peptone	2.0
NaCl	5.0
K_2HPO_4	0.3
Agar	5.0
Bromothymol Blue	0.08
pH	7.1

The medium was autoclaved at 10 psi for 20 min. Glucose (10%) solution was autoclaved separately at 10 psi for 20 min and was added to the basal medium to achieve a final concentration of 1%. About 5 ml of the medium was poured in sterile tubes. The isolates were stabbed, in duplicates from 18 to 24 h old LB broth cultures. One of the stabs was covered with paraffin oil, (sterilized prior to use in oven at 80^{o}C in oven for 2 d), to check the fermentation mode, and other was to check oxidation mode. Both the tubes were incubated at 30^{o}C for 2 to 4 d. Control tubes with and without carbohydrate and without culture, were uninoculated for comparing the change in colour.

2.2.4.5 Arginine Dihydrolase test:

Pseudomonas spp. give arginine dihydrolase test positive as these organisms possess characteristic enzyme arginine dihydrolase, which liberates ammonia when the culture is grown in the presence of arginine. Ammonia released may have deleterious effect on plant pathogens and helps in PGPR activity. The production of ammonia was checked using dye bromocresol purple which is yellow in acidic condition and purple in basic condition so if organism produces ammonia then color changes to purple.

Medium for Arginine Dihydrolase test:

Ingredients	(g/l)
Peptone	5.0
Beef extract	4.0
Bromocresol purple	0.1
Cresol red	0.005

Glucose	0.5
D/W	1000 ml

About 5 ml of above mentioned medium was dispensed in tubes and autoclaved at 15 psi for 15 minute. Arginine (1%) was autoclaved separately and added to the tubes. The isolates were inoculated in duplicates, one tube with arginine and other without it, and covered with paraffin oil, to prevent false positive results. The cultures were allowed to grow overnight. Next day the colour change was observed and compared with uninoculated tubes as well as tubes without arginine acting as control, to monitor the change.

2.2.5 Identification of isolates by molecular method:

Molecular technique was based on presence of conserved DNA sequences in the isolates which are hallmarks of fluorescent *Pseudomonas* spp. PCR was carried out using primers specific for 16S-23S rRNA Internal Transcribed Spacer (ITS) of *Pseudomonas* (Locatelli et al., 2002), which amplified the region including the 3' half of the 16S rRNA gene with the whole 16S-23S rRNA ITS sequence giving an expected amplicon size of 560 bp.

2.2.5.1. Genomic DNA isolation from isolated strains:

DNA isolation of samples was carried out by modified cetyl trimethyl ammonium bromide CTAB method. About1.5 ml of overnight grown cultures was centrifuged at 10,000g for 5 min at 4^0C. The supernatant was drained off and the pellet was resuspended in 200 µl of Tris EDTA ($T_{10}E_{0.1}$) (10 mM Tris HCl with 1mM EDTA at pH 8.0). It was vortexed vigorously to resuspend the pellet and then was kept at 60^0C for 30 min and 100µl of NaCl (3M) along with 80 µl of CTAB mixture (10% CTAB in 0.7 M NaCl) were added to it. The contents were mixed thoroughly and incubated at 60^0C for 10 min. Equal volume of phenol-chloroform-isoamyl alcohol mixture in the ratio 25:24:1 was added and centrifuged at 10,000 g for 12 min at 4^0C. The aqueous phase was collected to it and 2-3 volume of chilled 100% ethanol was added and kept overnight. The DNA precipitates were recovered by centrifugation at 10,000g for 10 min at 4^oC. The supernatant was drained off and the pellet was washed twicely with 70% ethanol and the pellet was dried. The DNA was resuspended in T_{10} $E_{0.1}$.

2.2.5.2 PCR amplification:

PCR amplification of fluorescent pseudomonas ITS was done by using specific Primers and Eppendorf thermo cycler.

Primer sequences were as follows

ITS1F 5' AAGTCGTAACAAGGTAG 3' (17 mer)

ITS2R 5' GACCATATATAACCCCAAG 3' (19 mer)

Primers were obtained from Sigma Aldrich, Mumbai, India.

The system used for PCR amplification is shown below.

Reactants	Quantity (µl)
10X Taq Buffer A (with 2.5 mM $MgCl_2$)	2
dNTPs mix(10 mM)	2
Primer 1(Forward) (2mM)	2
Primer 2 (Reverse) (2mM)	2
Template DNA (50 ng/ µl)	1
Taq polymerase (3U/µl)	1
Milli Q sterilized water	10
Total	20µl

The amplified DNA fragments were analysed by polyacrylamide gel electrophoresis as described below. PCR was performed as per the protocol described in Fig. 2.5.Amplified fragments were resolved on 1% agarose gels at 60 mV by standard protocol (Sambrook et al., 2001). A 100 bp ladder DNA marker was used as a size standard.

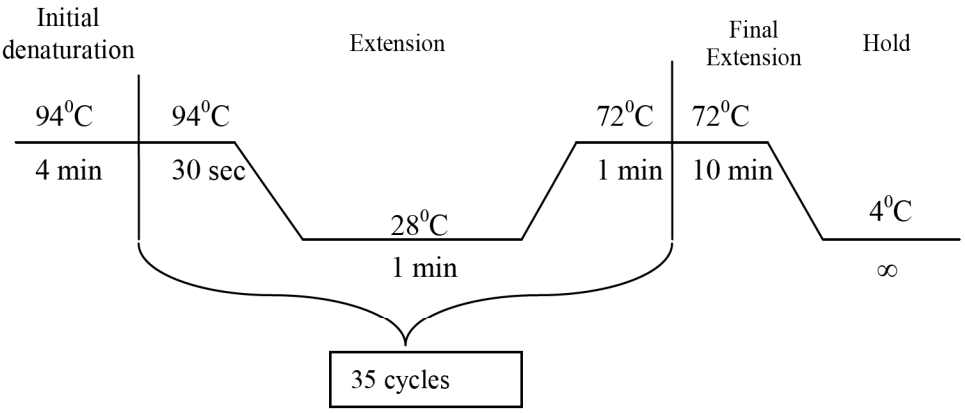


Fig 2.5 Schematic representation of the steps in PCR amplification of ITS

2.2.6 Characterization of fluorescent pseudomonad strains based on PGPR traits:

2.2.6.1 Siderophore production:

Siderophore production was checked, using Chrome Azurol – S (CAS) agar plates as described by Schwyn and Neilands (1987). The basic principle underlying the test is that when a strong ligand L (e.g. siderophore) is added to highly coloured dye-Fe^{3+} complex, the iron-ligand complex is formed and the release of free dye is accompanied by a colour change.

$$Fe\text{-}Dye^{3-\lambda} + L^{x-} \longrightarrow FeL^{3-x} + Dye\lambda^{-}$$

Chrome Azurol – S (CAS) agar plates as described by Schwyn and Neilands (1987)were prepared using the following solutions.

Dye solution (100ml): 60.5 mg CAS dye was dissolved in 50 ml D/W and mixed with 10 ml iron solution (1mM FeCl3 in 10 mM HCl). Prepared solution was added slowly to 72.9 mg hexadecyl trimethyl ammonium (HDTMA) dissolved in 40 ml distilled water. The resulting dark blue dye solution was autoclaved at 15 psi for 15 min.

10X MM9 salts (100ml): MM9 growth medium was prepared from M9 by reducing concentration of phosphate salts to 0.03% KH_2PO_4.

Composition of 10X MM9 salts:

Ingredients	(g/100ml)
$Na_2HPO_4.7H_2O$	0.15
KH_2PO_4	0.15
NaCl	0.5
NH_4Cl	1.0
D/W	100 ml

The medium was autoclaved at 10 psi for 20 min. To autoclaved cooled medium the following solutions, that were prepared and autoclaved separately (10 psi for 20 min), were added.

1 M $MgSO_4.7H_2O$	1 ml
0.1 M $CaCl_2$	1 ml

100ml blue agar: To 75 ml double distilled water, 10 ml 10X MM9 salts, 3.024 g 1.4-piperazine diethane sulfonic acid (PIPES) and 1-2 g of 50% (w/w) NaOH solution were added so as to obtain a pH of 6.8. After dissolution of the salts, 2.5 g of agar was added and the medium autoclaved at 15 psi for 15 min. After cooling to 50°C, 3ml of casamino acids (10%), 1ml glucose (20%), 0.5ml L – glutamic acid (10%), and 0.25 ml biotin (0.02%) were added followed by 10ml dye solution

which was added and mixed uniformly without allowing foam formation and was poured in Petri plates. For detection of siderophores, freshly overnight-grown culture was pellet down by centrifugation at 5000 RPM for 5 min., washed with sterile saline, resuspended in saline and then spotted on the plate. The colonies developed were observed for yellowish orange halo around them which would be formed if the organism produced siderophore because Fe-CAS-HDTMA complex which is blue in color would be converted to yellowish orange color due to the liberation of iron and free CAS dye around the colonies. The CAS agar contains 0.1M PIPES buffer that prevents pH change during growth. HDTMA a detergent serves the purpose of achieving intense color i.e. it improves the sensitivity of the reaction. The colony zone / colony size of various isolates were calculated was compared to the model strain *Pf* CHA0.

2.2.6.2 Indole acetic acid (IAA) production:

Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986).

Cells were grown in the following minimal medium.

Ingredient	(g/100ml)
Na_2HPO_4	0.6
KH_2PO_4	0.3
NaCl	0.050
NH_4Cl	0.1
D/W	100 ml

The medium was autoclaved at 10psi for 20 minutes.

1M MgSO4	0.2 ml
20% Glucose	2 ml
0.1M $CaCl_2$	100µl

Separately prepared solutions of 1M $MgSO_4$, 20% glucose and 0.1 M $CaCl_2$ were added to the autoclaved basal medium as mentioned above. Λ loopful of overnight grown bacterial culture was inoculated in 2ml minimal medium amended with out and with 50 µg/ml tryptophan. Cultures incubated for 48-72 h at 30°C on rotary shaker. IAA production was detected by the modified method as described by Brick et al., 1991. Grown culture was centrifuged at 10,000 RPM for 15min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml of 35% perchloric acid with 1ml of 0.5% $FeCl_3$). Samples were incubated for 25 min at room temperature (RT), OD_{530nm} was measured with the help of UV-Vis

spectrophotometer (Shimadzu Co. Japan). IAA standard solution (Hi-media ltd., India) was prepared at 100 µg/ml in 50% ethanol. IAA quantification values were recorded by preparing calibration curve made by using IAA standard in the range of 10-100 µg/ml.

2.2.6.3 HCN production:

HCN is a volatile compound produced by many fluorescent pseudomonads. HCN production by isolates was checked by method of Bakker and Schipper (1987) on King's B medium amended with 4.4 g/l glycine. Cyanogenesis from glycine results in the production of HCN, which is volatile in nature. A single isolate was streaked on each plate. A Whatman no. 1 filter paper disc (9 cm in diameter) was soaked in 0.5% picric acid in 2% sodium carbonate prepared in autoclaved D/W. The soaked disc was attached on to the lid of each inoculated petriplate and the plates were sealed with parafilm and incubated at 30^{o}C for 3- 4 d. An uninoculated medium with the soaked filter paper was kept as control for comparison of results. After incubation period, the change in colour of the filter paper was checked. Brownish colour indicated production of HCN. Reaction of HCN with picric acid in presence of Na_2CO_3 results in the colour change in picric acid soaked filter paper from deep yellow to orange brown. In the case of negative test, the deep yellow color of the filter paper remains unchanged.

2.2.7 Characterization of fluorescent pseudomonad strains for lytic enzymes:

Many biocontrol organisms produce cell-wall degrading enzymes and other hydrolases that are antagonistic to fungal phytopathogens. Protease, cellulase and chitinase are most important among the lytic enzymes and were assayed as follows.

2.2.7.1 Protease production:

The medium for checking protease production was Luria–Bertani (LB) agar medium to which 1% skimmed milk was added. Protease positive strains were recorded as showing a zone of clearance around the site of growth. Autoclaved LB agar medium was supplemented with sterile skimmed milk solution when molten LB agar was about 45^{o}C to prevent protein coagulation. Overnight grown cultures were inoculated on the media plate and incubated for 48 h. *Pf* CHA0 has been reported to produce extracellular protease (Siddiqui et al., 2005) was used for comparison.

2.2.7.2 Chitinase Activity:

Chitin is a polymer of N-Acetyl Glucosamine (NAG) and endochitinase activity (hydrolytic splitting within chitin polymer) was checked using Monreal Reese (MR) medium. Its composition is as follows.

MR Media:

Ingredients	(g/l)
Chitin	5.0
$(NH_4)_2SO_4$	1.0
Yeast Extract	0.5
$MgSO_4$	0.3
KH_2PO_4	1.36
Agar	25
D/W	1000 ml

Chitin was used as a semi liquid paste. The medium was autoclaved at 15 psi for 15 min and overnight cultures were streaked on the MR media plates and were incubated for 7 day after which growth of isolates was checked.

2.2.7.3 Cellulase Activity:

Cellulose, the glucose polymer with α-1, 3- linkage, is an important constituent of cell wall of fungi. Carboxy Methyl Cellulose (CMC) was used as the carbon source. CMC which is water-soluble was prepared in 50 mM sodium-phosphate buffer (pH 7). The medium for detection of cellulose degrading activity was as follows.

Medium composition:

Ingredients	(g/100 ml)
Yeast Extract	1.0
CMC	0.1
Peptone	0.05
Agar	1.5

Medium was autoclaved at 15 psi for 15 min.

Overnight grown fresh cultures were inoculated on the plates and incubated at 30^0C for 2 d. Staining was done with 1% Congo red. After incubation period, plate was flooded with Congo red and destained by washing with water. Unstained area around site of inoculums indicated degradation of cellulose and thus is considered positive for the test.

2.2.8 Bioassay for 2, 4 DAPG:

Among antifungal metabolites produced by fluorescent pseudomonas, 2, and 4 - DAPG has inhibitory effect on methicillin resistant *Staphylococcus aureus ATCC 6538*. In order to screen for

2,4 - DAPG producers from the isolates which had shown shown antifungal activity, *S. aureus ATCC 6538* was used as sensitive strain for bioassay of 2,4 - DAPG. Here the overnight grown S. aureus was plated on Luria-Bertanni Agar plates. Wells (1cm) were bored using cup borers and 80 µL of 0.22 u filtered, 3-4 days grown cultures of fluorescent pseudomonad in KMB broth were poured in the wells. The plates were incubated at 37°C for 24 h and were monitored for the zone of inhibition around the wells.

2.2.9 Characterization of PGPR strains for 2-keto-4-methylthiobutyric acid (KMBA) pathway:

The presence of KMBA pathway was determined by formation of precipitation with 2, 4-dinitrophenylhydrazine according to Primrose (1977). The culture medium was separated from the cells by 10,000 rpm at 4^0C. To 10 ml of culture supernatant, 1 ml of 0.1% 2, 4-dinitrophenylhydrazine in 2 M Hcl was added and stirred at RT in total darkness. In the presence of KMBA a yellow precipitate was formed after 30min.

2.2.9.1 Preparation of bacterial extracts for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase assay:

ACC deaminase activity was measured in bacterial extracts prepared in the following manner. Bacterial cell pellets were each suspended in 1 ml of 0.1 M Tris-HCl, pH 7.6 and transferred to a 1.5 ml micro centrifuge tube. The contents were centrifuged at 16,000x*g* for 5 min in a micro centrifuge and the supernatant was removed with a fine-tip transfer pipette. The pellet was suspended in 600µl of 0.1 M Tris-HCl, pH 8.5. 30µl of toluene was added to the cell suspension and vortexes at the highest setting for 30 s. At this point, a 100 µl aliquot of the "toluenized cells" was set aside and stored at 4°C for protein assay at a later time. The remaining toluenized cell suspension was immediately assayed for ACC delaminate activity.

2.2.9.2 Assay of ACC Deaminase activity:

ACC deaminase activity was assayed according to the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate released when the enzyme, ACC deaminase, cleaves ACC. The nmole of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of *a*- ketobutyrate ranging between 0.1 and 1.0 nmol. A stock solution of 100 mM α -ketobutyrate (Sigma-Aldrich Co.) was prepared in 0.1 M Tris-HCl pH 8.5 and stored at 4°C. Just prior to use, the stock solution was diluted with the same buffer to make a 10-mM solution from which a standard concentration

curve was generated. A series of known concentrations of *a*-ketobutyrate were prepared in a glass test tube (100 x 13 mm); each point in the series was assayed in duplicate. To this 300 ul 2,4-dinitrophenylhydrazine (DNPH) reagent (0.2 % 2,4-dinitrophenyl-hydrazine in 2 N HCl; Sigma-Aldrich Co.) was added and the contents were vortexed and incubated at 30°C for 30 min during which time the *a*-ketobutyrate was derivative as a phenylhydrazone. The color of the phenylhydrazone was developed by the addition of 2.0 ml of 2 N NaOH; after mixing, the absorbance of the mixture was measured at 540 nm.

The absorbance of the assay reagents including the substrate ACC and the bacterial extract were taken into account by setting up appropriate controls. After the indicated incubations, the absorbance at 540 nm of the assay reagents in the presence of ACC was used as a reference for the spectrophotometric readings; it is subtracted from the absorbance of the bacterial extract plus the assay reagents in the presence of ACC. The contribution of the extract, i.e., the absorbance at 540 nm of extract and the assay reagents without ACC, is determined and subtracted from the absorbance value calculated above. This value was used to calculate the amount of α-ketobutyrate generated by the activity of ACC deaminase.

All sample measurements was carried out in duplicate. Two hundred µl of the toluenized cells were placed in a fresh 1.5-ml micro centrifuge tube; 20µl of 0.5M ACC was added to the suspension briefly vortexes and then incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56 N HCl, the mixture is vortexes and centrifuged for 5 min at 16,000x*g* in a micro centrifuge at RT. One ml of the supernatant was vortexes together with 800µl of 0.56 N HCl in a clean glass tube (100x13 mm). There upon, 300 µl of the DNPH reagent was added to the glass tube, the contents vortexes and then incubated at 30 °C for 30 min. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance of the mixture was measured at 540 nm.

2.2.10 Restriction Fragment Length Polymorphism of ITS amplicon:

2.2. 10.1. Restriction endonuclease (RE) digestion of PCR products:

When the ITS-PCR products were digested with *HaeIII* and *MspI* , Hae III was found to be effective cutter so it was selected for further study. The sum of the sizes of restriction fragments agreed with the sum of the sizes of undigested ITS amplification product. The *Hae*III, restriction patterns of the amplified rDNA ITS regions were used for cluster analysis. Fragments of over 100

bp were included for calculating similarity matrices. The buffers and the restriction enzymes were obtained from Fermentas, India. Following systems were prepared for digestion of PCR products:

PCR reaction mixture	10µl (~0.1µg DNA).
Water (nuclease free)	18µl
10X recommended buffer	2µl
Restriction endonuclease (0.5 U)	1-2µl

The contents were placed in a 0.5ml capacity eppendorf tube, mixed gently and typically incubated at 37°C for 16 h.

An 8% gel was prepared, that is 8% of 30% acrylamide. For 10 ml of gel mixture that was to be prepared, 30% acrylamide /bis acrylamide - 2.66 ml; 0.5 M Tris acetate EDTA pH- 6.8 (TAE) buffer -,7.44 ml ratio of acryl amide and bis acryl amide were added. The SDS-PAGE was run for about 3 hours at 60mV. In order to observe the bands in the gel, silver staining was done. Gel was carefully removed with gloves and rinsed with distilled water. Methanol (20% v/v in water) was added and kept for 10 min. The methanol was drained and this was repeated again. This step was followed by a short wash with water (Methanol reduces the pore size and O/N incubation should be avoided if the DNA is of small size). About 50 ml of 0.7% HNO_3 was added (per gel) and incubated for 3 min. This was followed by short rinses with water. This step was done twice. About 50 ml of 0.2% $AgNO_3$ was 1-2 minute. A short water wash (of 30 s) was done after this step. About 100ml of developer solution was added (per gel) and it was constantly shaken till the bands appeared and the background ratio was maximum. Composition of developer is as follows: 2.29% of Na_2CO_3 + 125 µl formaldehyde + 20µl of Sodium thiosulphate from 20mg/ml of stock solution. The developer solution was prepared freshly and as soon as the bands appeared, the developer solution was removed and about 250 ml of 3% glacial acetic acid was added to stop the reaction. The gel was washed with 20% methanol and stored.

2.3 RESULTS AND DISCUSSION:

2.3.1 Isolation of plant growth promoting fluorescent strains by enrichment:

Three rounds of plant inoculations were carried out to obtain efficient root colonizing fluorescent pseudomonad. In the rhizosphere root exudates are the primary source of nutrients and root colonizer is well adapted to the utilization of nutrients (Van Overbeek and Van Elsas, 1995). To obtain isolates surviving on root exudates the Murashige and Skoog media used for plant growth was not supplemented with any carbon source, only the isolates survive and thrive on the root exudates would be enriched. After each round of plant inoculation the plants were assessed for their shoot root length and were compared with standard strain *Pf* CHA0 and the controls where no culture was added and only those isolates which showed better growth than control and similar to or better than *Pf* CHA0 were selected for further studies. Fig. 2.6 shows the representative plantlets with varying effect of bacterial inoculation on root length. Those showing negative effect (for e.g. last panel in Fig. 2.6) were not considered for next round of plant assay. Motile rhizobacteria may colonize the rhizotic zones of plant more profusely than non-motile organisms resulting in better rhizosphere activity (Weller, 1988). So those isolates that would have succeeded to reach the root tip at the end of 7 d of incubation would be good root colonizers. After every round 1 cm of root tips were cut and its suspensions were used for seedling incubation in the next round. Fig. 2.7 shows representative isolates obtained from the root tips of the plants. Isolation of bacteria tightly associated with plant roots was ascertained by vigorous shaking which helped for screening efficient colonizers as only those will be adhered to the roots. Use of antibiotics in the protocol of screening fluorescent pseudomonad has made the screening procedure much less tedious and time saving as much of gram positive and eukaryotes, sensitive to these antibiotics, will not grow on the media supplemented with these antibiotics. The fluorescence levels in different isolates were different. The isolates from ground nut and sugarcane showed very high fluorescence, while isolates from cotton showed very less fluorescence. From three cycles of plant inoculation studies nearly 83 isolates were obtained from various plant rhizospheres, which were screened based on fluorescent colonies (Fig.2.7).

2.3.2 Characterization of fluorescent bacterial isolates for the antifungal activity:

The fungal growth inhibition by rhizospheric isolates was assessed by measuring the inhibition of mycelia radial growth in the dual culture plate assay against the fungus *Rhizoctonia bataticola*

(Fig. 2.8). Inhibition was quantitated in terms of the distance traveled by the fungus towards the culture and was compared to *Pf* CHA0. Less growth of fungal mycelia towards the culture indicates more antifungal activity of the isolate (Table 2.1).

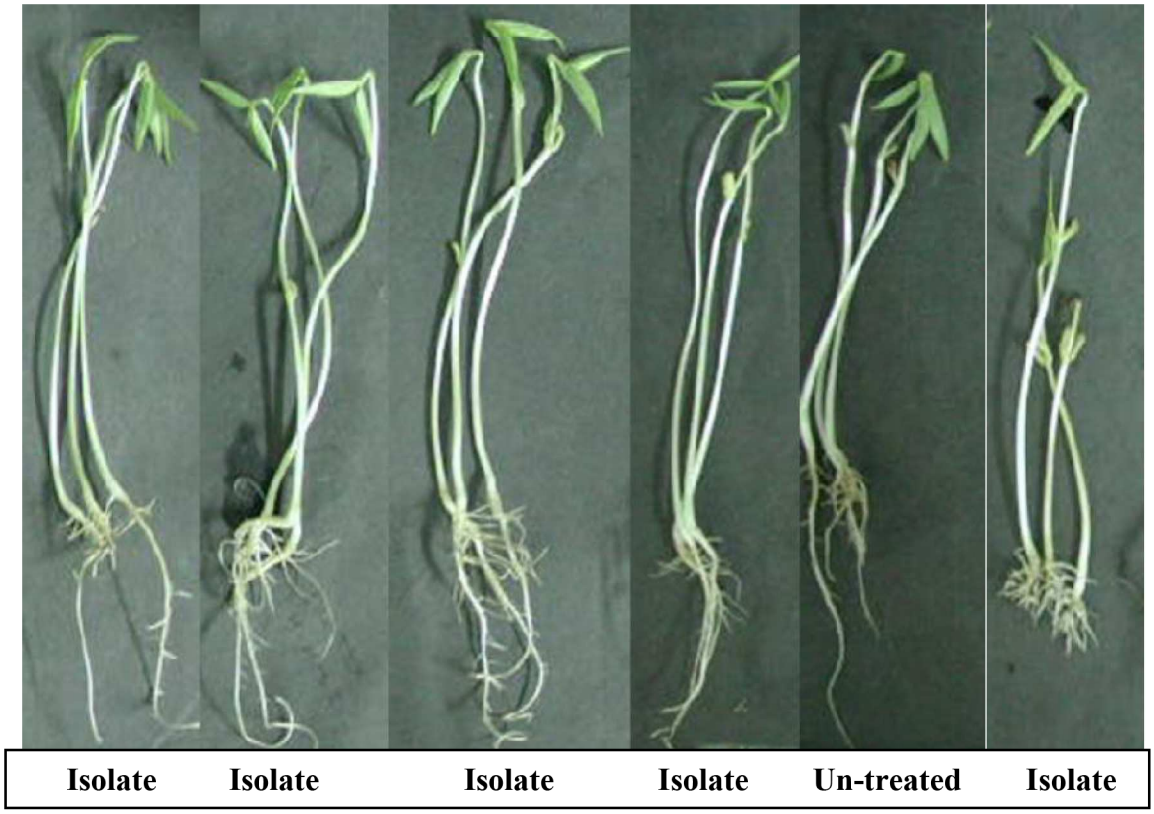
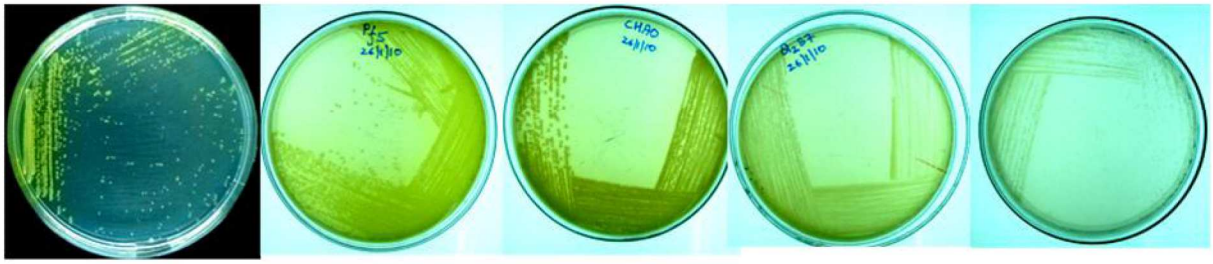


Fig. 2.6 Representative plants from the enrichment assay



(G2) (Pf-5) (CHA0) (Q287) (G6)

Fig. 2.7 Representative strains of fluorescent pseudomonad on Kings B plates

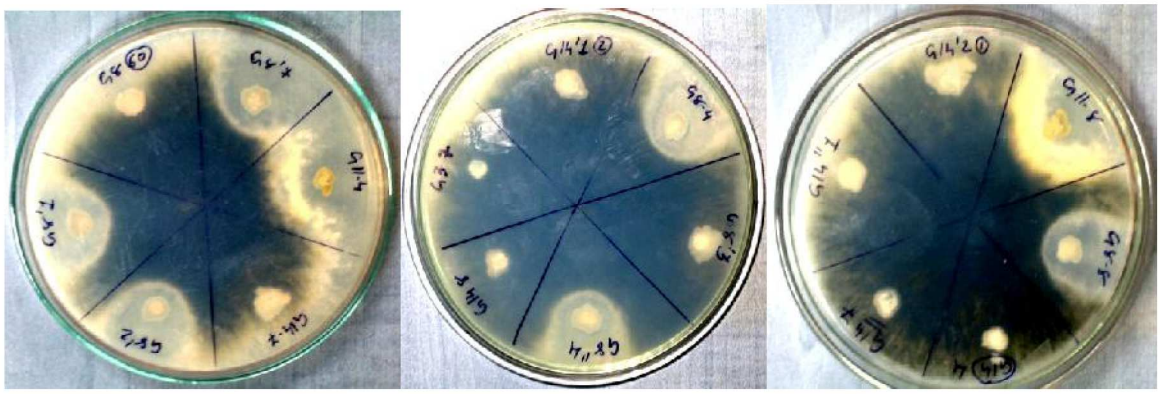


Fig.2.8 Antifungal activity against *R. bataticola* by representative fluorescent isolates

Radar graphs (Fig. 2.9) depict the antifungal activity of isolates, these isolates have maintained zone of inhibition while fungus had outgrown *Pf* CHA0. Of 83 isolates, those with moderate-to-good antifungal activity were screened by in vitro antifungal assay against the fungus *Rhizoctonia bataticola.* All the isolates which showed antifungal activity better than *Pf* CHA0 or similar to *Pf* CHA0 were considered as good isolates because they can compete in rhizosphere with phytopathogens better than *Pf* CHA0. Maximum numbers of strains having higher antifungal activity have been obtained from groundnut rhizosphere followed by cotton and sugarcane rhizosphere (Table 2.2).

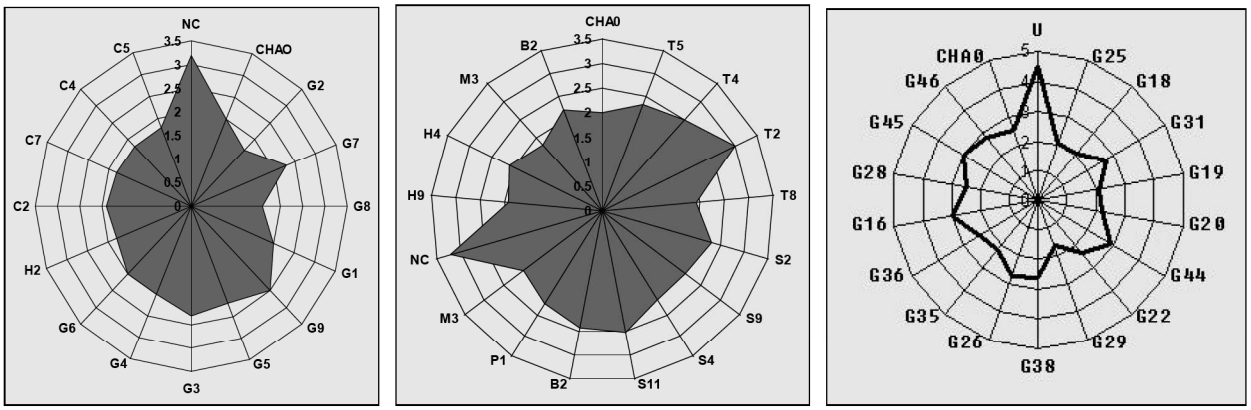


Fig 2.9 Radar plots for the inhibition of *R. bataticola* by fluorescent bacterial isolates (U-Uniinoculated, NC- Negative control (uninoculated)

Isolates like G19, G20 and G25 have shown antifungal activity much better than *Pf* CHA0 as even after 7 days of incubation (Table 2.1).

Table 2.1 Antifungal activity of fluorescent bacterial isolates

Strain	Fungal Inhibition (%)		Strain	Fungal Inhibition (%)
G8	64.60		M3	44.44
G29	64.40		G4	44.40
C5	56.00		G1	44.40
G 18	55.55		G38	42.22
G25	55.55		S4	41.20
G2	54.00		S9	41.20
G19	53.33		H4	41.22
G20	51.11		G31	40.00
H2	50.88		G26	40.00
C7	50.80		P1	38.00
C4	50.80		G 16	33.33
G22	48.88		G44	35.55
G36	48.88		G 45	33.33
C2	47.60		G46	37.77
T8	47.66		G7	34.80
H9	47.66		G3	34.80
G6	45.60		G5	32.00
CHA0	44.44		B2	31.60
G28	44.44		G9	28.40

(G-groundnut, C-Cotton, T-tomato, B-Banana, P-Potato, M-Maize, S-Sugarcane, H-harvested soil)

Table 2.2 Enumeration of isolates based on antifungal activity

	Tobacco	Sugar cane	Rice	Pigeon Pea	Banana	Maize	Cotton	Ground nut
Fluorescent strains	7	9	4	3	9	3	4	44
Antifungal positive strains:	4	4	2	1	2	1	3	26
Antifungal activity better than *Pf* CHA0	1	0	1	0	0	0	3	14
Antifungal activity similar to CHA0:	0	2	1	0	0	1	0	3

2.3.3 Characterization of fluorescent isolates for the P-solubilization:

Most of the reports on solubilization of mineral phosphates by microorganisms suggest that organic acid excreted as consequence of primary metabolism to be responsible for P solubilization (Bajpai & Sundararao, 1971; Gyaneshwar et al., 2002). These organic acids through their hydroxyl and carboxyl groups chelate the cations bound to phosphate and release soluble phosphate (Kpomblekou and Tabatabai, 1994). P solubilization was checked on Pikovaskya′ s agar (Dicalcium – Phosphate Agar), clearance zones around the cultures inoculated was due to solubilizaton of inorganic phosphate (Ca-P) presumably by organic acids produced by isolates. Phosphate solubilisation ability of organisms was semi-quantified in terms of C_Z / C_S ratio and was compared with standard strain *Pf* CHA0 (Fig. 2.10, Tables 2.3 & 2.4).

Based on both P- solubilization and antifungal activity strains have been categorized in three classes. Isolates having good antifungal activity as well as good P- solubilisation ability similar to standard strain *Pf* CHA0 (G16, G18, G22, G26, G29, G31, G35, G36, G38, G45, G46, T6, S4, S9, P1, P2, P3, C4, G1, G2, and G8); isolates having good antifungal activity but weak P-solubilisation activity (G19, G20, G23, G25, G28 and G44) and those that did not show antifungal activity but have shown P- solubilisation ability (G1, G3, G4, G5, G9, G11, G14, G40, G41, G42 and G43).

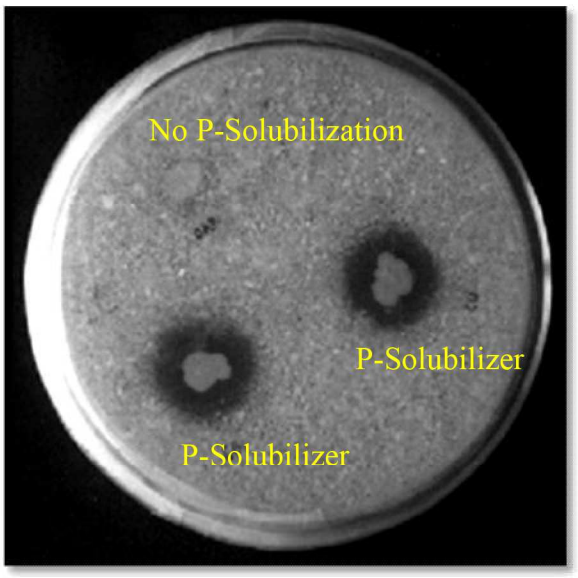


Fig. 2.10 Representative diagram of P-solubilization by fluorescent bacterial isolates

Table 2.3 Phosphate solubilization ability of fluorescent bacterial isolates

Isolates	P-Solubilization		Isolates	P-Solubilization
G 1	+		G 23	-
G 2	-		G 24	-
G 3	+		G 25	+
G 4	+		G 26	+++
G 5	+		G 27	-
G 6	-		G 28	-
G 7	-		G 29	+++
G 8	-		G 30	-
G 9	+		G 31	++
G 10	-		G 32	-
G 11	++		G 33	-
G 12	-		G 34	-
G 13	-		G 35	-
G 14	++		G 36	-
G 15	-		G 37	-
G 16	+		G 38	++

G 17	-		G 39	+
G 18	+++		G 40	+
G 19	+		G 41	+
G 20	-		G 42	++
G 21	+		G 43	-
G 22	+++		G 44	-
G 46	+++		G 45	+++

(+++ indicates high P-solubilizing activity, ++ moderate, + low
and – indicates no P- solubilizing activity)

Table 2.4 Enumeration of fluorescent isolates based on phosphate solubilization ability

Rhizosphere	Tobacco	Sugar cane	Rice	Pigeon Pea	Banana	Maize	Cotton	Ground nut
Phosphate Solubilizers	7	9	4	3	2	3	4	8
Better than Pf CHA0	2	7	2	3	0	3	3	3
Similar to CHA0	1	0	1	0	1	0	1	1

2.3.4 Siderophore production by fluorescent bacterial isolates:

The fluorescent pigment produced by *Pseudomonas* spp. is a powerful iron (III) scavenger and an efficient iron transporter. Siderophore production helps in better survival and competence of isolates and also acts as biocontrol against plant pathogens (fungal and bacterial) in iron deficient conditions. Siderophore production by rhizosphere microbes also helps in iron acquisition of plant in mineral deficient conditions as some plants are reported to be capable of obtaining iron from microbial siderophores (O'Sullivan and O'Gara, 1992). Soil pseudomonad produce fluorescent siderophores with both hydroxamate and phenolate groups; these siderophores have been classified as either pyoverdins or pseudobactins. Because Pvd-producing *Pseudomonas* spp. inhibit the growth of phytopthogenic fungi, it was possible that the antifungal activity of bacterial isolates might be siderophore mediated. Thus it was important to study siderophore production by

the isolates. As detected by a universal siderophore detection protocol using Chrome Azurol – S (CAS) agar described by Schwyn and Neilands (1987), a yellow halo was seen around the growth of bacterial isolates (Fig. 2.11). From tobacco rhizosphere isolates T2, T4, T8, T11, T12 have shown better siderophore production than CHA0 (Fig. 2.12). Isolates T5 has shown similar siderophore production than *Pf* CHA0 while T6 has shown poor production. Table 2.5 shows the summary of siderophore production by various rhizoshperic isolates. Soil pseudomonads produce fluorescent siderophores with both a hydroxamate and catecholate groups. A peak between 420-450 nm and 495nm indicated the presence of ferric hydroxymate and ferric catecholate, respectively.

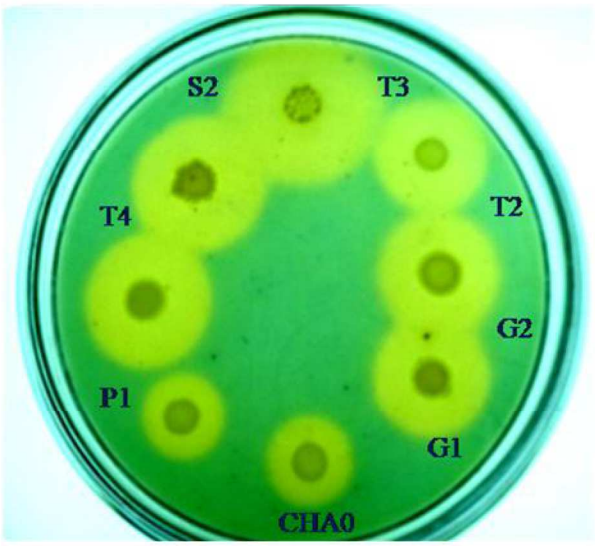


Fig. 2.11 Fluorescent pseudomonad isolates showing siderophore production on CAS plates

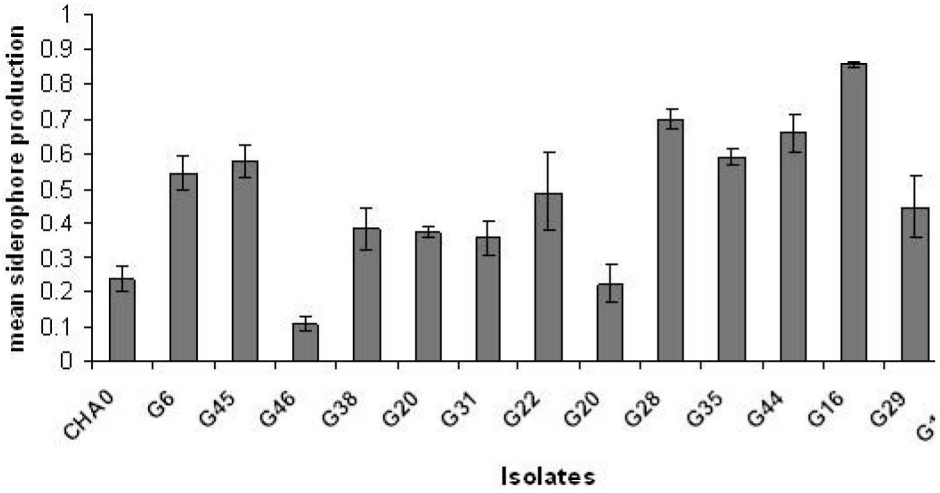


Fig. 2.12 Siderophore production by fluorescent bacterial isolates (Bars indicate S.D., n=3)

Table 2.5 Enumeration of fluorescent isolates based on siderophore production ability

Rhizosphere	Siderophore producers	Siderophore production higher than *Pf* CHA0	Siderophore production similar to *Pf* CHA0
Tobacco	7	5	2
Sugar cane	9	2	2
Rice	4	3	0
Pigeon Pea	3	1	1
Banana	2	2	0
Maize	3	2	1
Cotton	4	4	0
Ground nut	9	0	2

2.3.5 Identification of isolates by biochemical tests:

Fluorescent pseudomonad strains were identified as Gram –ve, rods or short rods, catalase positive, oxidase positive, arginine dihydrolase positive and Hugh-Leifson's O-F test (Oxidation mode present, fermentation mode absent) (Table 2.6),

Table 2.6 Number of fluorescent isolates from different rhizosphere samples positive for biochemical tests specific for fluorescent pseudomonad

	Ground nut	Sugar cane	Tobacco	Rice	Pigeon-Pea	Banana	Maize	Cotton	Brinjal
Total fluorescent strains	44	40	26	23	6	11	24	22	4
Catalase positive	41	38	26	20	5	10	24	20	4
Oxidase positive	38	35	20	15	4	8	20	18	1
Oxid./Ferment. test	26	20	18	15	4	5	16	12	0
Arginine dihydrolase test	9	12	15	9	4	3	3	7	0
Gram test	9	12	15	9	4	3	3	7	0
Fluorescent pseudomonad	9	12	15	15	4	3	3	7	0

2.3.6 Identification of fluorescent pseudomonad strains by molecular method:

Modified CTAB method yield genomic DNA in the range of 8-20 ng/ul genomic in case of various isolates (Fig. 2.13). The ITS primers are expected to give amplicon size of 560 bp and isolates showing amplicon size 560 bp were considered to be fluorescent *Pseudomonas* spp. (Fig. 2.14). Out of these 60 isolates 40 isolates which showed ITS positive were used in further characterization studies. Diversity Indices in each sample represents the absolute frequency (number of individuals) of each morphological category and is calculated by corresponding relative frequency (pi) of each morphological category by dividing each absolute frequency by the total number of organisms in the sample. After the identification of isolates by biochemical and molecular methods, Shannon index of diversity was derived from these relative frequencies (Zar et al., 1999) of the fluorescent pseudomonas in each sample (which indicates the population density of fluorescent pseudomonas) diversity was high in pigeon pea (0.5) and tobacco rhizosphere (0.269), but highest number of fluorescent pseudomonads were obtained from tobacco, rice, sugarcane and groundnut rhizosphere (Table 2.7).

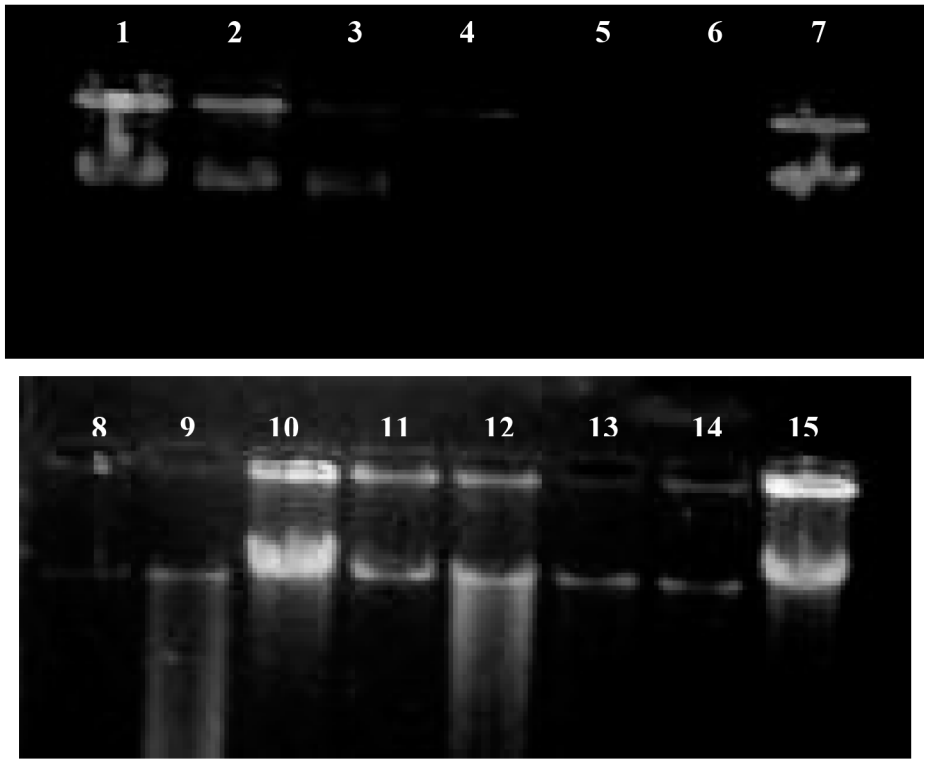


Fig. 2.13 Genomic DNA of representative fluorescent bacterial isolates (1-CHAO; 2- Pf5; 3-Q287; 4- G36; 5-P1; 6- G22; 7- H9; 8-C2; 9- G14; 10- G45;11- G46; 12- G5; 13- G25; 14- G6; 15- G30)

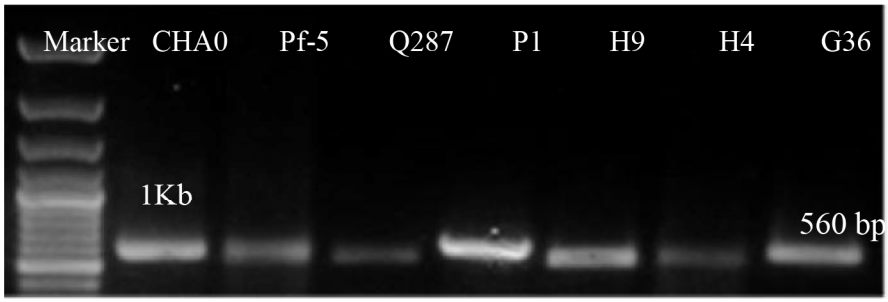


Fig. 2.14 (A)

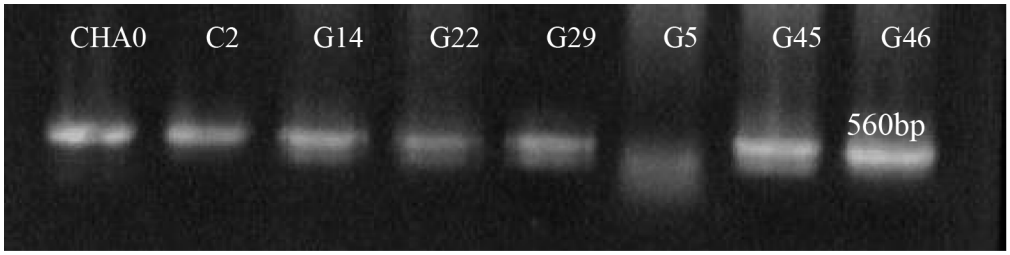


Fig.2.14 (B)

Fig.2.14 Amplification of fluorescent pseudomonad specific ITS (560bp) in the bacterial isolates

Table.2.7 Summary of biochemical and molecular positive strains of fluorescent *Pseudomonas* isolated from different rhizospheric samples

Rhizosphere sample	Arginine Dihydrolase	Gram staining	ITS amplification	Diversity Index	Selected strains
Ground nut	9	9	9	0.204	G1, G2, G3, G4, G5, G6, G7, G8, G9
Sugar cane	12	12	9	0.225	S1, S2, S4, S5, S6, S8, S9, S10, S11
Tobacco	15	15	7	0.269	T2, T4, T5, T6, T8, T11,T12
Rice	9	9	4	0.174	H2, H4, H8, H9
Pigeon Pea	4	4	3	0.5	P1, P2, P4
Banana	3	3	2	0.181	B2, B3
Maize	3	3	3	0.125	M1, M2, M3
Cotton	7	7	4	0.181	C2, C4, C5, C7
Brinjal	0	0	0	-	-

2.3.7 Characterization of fluorescent pseudomonad for indole acetic acid (IAA) production:

The aggressive root colonization and defensive retention of rhizosphere niches by bacteria are enabled by the PGPR traits like siderophore production, production of antibiotics, phosphate solubilization, and phytostimulation (Bloemberg et al., 2001, Bais et al., 2004). Isolates were

characterized for IAA production. Inoculation of canola seeds with IAA producing *Pseudomonas putida* GR12-2 resulted in 2 - or - 3 fold increases in the length of seedling roots (Caron et al., 1995). IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. It is possible to say IAA and ACC deaminase work in concert to stimulate root elongation. Exogenous IAA is known to increase activity of ACC synthase (Peck and Kende, 1995), which catalyzes production of ACC in plants. Bacterial isolates showing good IAA production can be putative phytostimulants increasing the root shoot length of the plants in which they are inoculated. It can be seen from the Table 2.8 that IAA production is between 10 – 22 µg/ml. G1, G3, C7, C2, P1, are isolates from ground nut, cotton, pigeon pea rhizospheres, showing better production than *Pf* CHA0, while other isolates are showing low production of IAA (Table 2.8). IAA production was checked in presence and absence of tryptophan (Fig. 2.15) as studied by Ahmed et al., 2005.

Table 2.8 IAA production by fluorescent *Pseudomonas* isolates (after 48 h)

Rhizospheric isolates	IAA production (µg/ml)
Pf CHA0	11.00
G4	12.60
G2	9.30
G1	20,0
G3	14.0
C7	15.6
P1	15.0
G6	11.6
H4	11.3
C2	22.0
M3	11.0

IAA production by bacterial isolates ranged from 25-31µg/ml in strains without supplementation of tryptophan as a precursor. With tryptophan supplementation, IAA production was found to range between 33-43µg/ml. Isolates G25 and G18 exhibit high IAA production in presence of tryptophan as compared to standard strain Pf-5 as seen in Fig. 2.15.

L-tryptophan is the common substrate of pyrrolnitrin and IAA biosynthetic pathways (Kirner et al., 1998; Loper, 1997), resulting in distribution of the common substrate in to the two pathways. The bacterial isolates are found to also show antifungal activity, so the possibility is there that there could be differences in the apportionment for utilization of L-tryptophan as precursor for antibiotic as well as IAA does exist, which might affect the production of IAA. The difference in IAA production by the different isolates may also be attributed to difference in the biosynthetic pathways preferred by different isolates. From tryptophan, indolepyruvic acid is produced via tryptophan transaminase which is the first step in IAA biosynthetic pathway, which is then

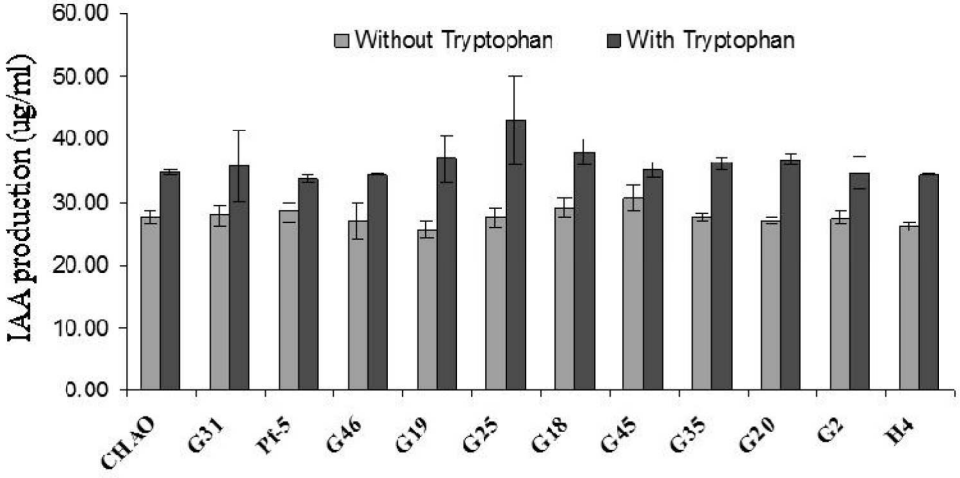


Fig. 2.15 IAA production by fluorescent *Pseudomonas* isolates (72-96h) (Bars indicate S.D., n=3)

followed by indolepyruvate decarboxylase (IPDC) for IAA production.The *ipdc* gene of *Pseudomonas putida* GR12-2 is not transcribed from the operon containing the other genes involved in biosynthesis of IAA by indolepyruvic acid pathway, but is transcribed from its own promoter and has a transcription termination sequence just downstream of the translation stop codon. It is reasonable to conclude that the enzymes involved in the indolepyruvic acid pathway are not expressed from an operon because multiple copies of the gene encoding the first enzyme in the pathway, an aromatic aminotransferases, are often present in a single bacterium, and the enzyme prefers amino substrate other than tryptophan (Kittel et al., 1989).

2.3.8 Study of other traits responsible for antibiosis in fluorescent Pseudomonads:

In P*seudomonas spp.* biocontrol activities mediated through production of antibiotics and also various lytic enzymes. This lytic enzyme mainly degrades the structural components of the

pathogenic organisms and thus protects plants from attack of the pathogenic organisms like fungi, nematodes, etc. Mainly the lytic enzymes secreted are protease, chitinase and cellulase.

2.3.8.1. Protease activity:

For antagonistic fungi, many biocontrol strains have been shown to produce extracellular proteases. The ability to degrade intracellular proteins has been attributed as an antifungal property. In strain *Pf* CHA0, the production of the major extracellular EDTA-sensitive protease has been reported (Siddiqui et al., 2005). Extracellular protease activity was determined qualitatively by inoculating loopful of overnight grown cultures on LA plates containing 1% of skimmed milk. Proteolysis was indicated by a zone of lyses (clearance) around the colony after 36 hrs at 28^{o} C. Majority of biocontrol isolates did not shown proteolytic activity as no zone of lysis was observed even after incubating for next 24-48 h. Few strains e.g. B2, C2, C5, H9, M3, G7, G30, G32 and G44 have shown protease activity but lesser than standard strain *Pf* CHA0 (Table 2.9).

2.3.8.2. Chitinase activity:

Chitin being the major cell-wall component of fungi, is targeted its antagonists. Several antifungal agents secrete chitinase enzyme and thus resists against fungal disease. Isolates were streaked on MR media where chitin is the only carbon source and kept for incubation for 7 d. isolates that show growth on this media are able to degrade chitin and thus are Chitinase positive while those which could not grow on this media are chitinase negative. Isolates like G35 and G36 have shown moderate growth on MR media after incubation period of 7 d (Table 2.9), while rests of the isolates are chitinase negative.

2.3.8.3 Cellulase activity:

Cellulolytic activity have occasionally been reported among fluorescent pseudomonad, but compared to the extensive work on these enzymes in other bacteria and microfungi, very little work has addressed their role in antagonistic *Pscudomonas* spp. Isolates which produce cellulase degrade CMC and a clear unstained zone will be seen around the isolate, after staining with Congo red, while those which do not show unstained zone are negative for the test. All the isolates were found to be negative for the cellulase activity (Table 2.9).Thus, although the isolates showed very good antifungal activity against the pathogenic fungus *R. bataticola,* majority of them have been found to be negative for the lytic enzyme production, which have been reported for their role in antagonistic effect against many pathogenic organisms (Table 2.9). Thus it could

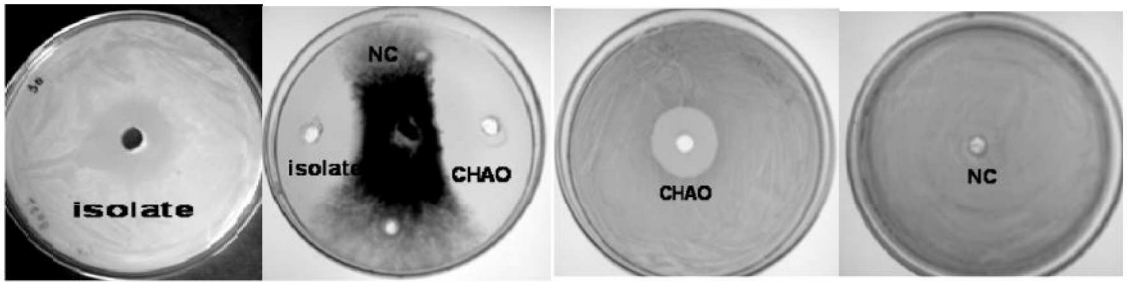
be concluded that these isolates must be producing other means of antagonism against the fungus for e.g. antibiotics.

Table 2.9: Production of lytic enzymes by fluorescent *Pseudomonas* strains

Strains	Protease	Chitinase	Cellulase		Strains	Protease	Chitinase	Cellulase
G 8	-	-	-		T8	-	-	-
G 12	-	-	-		S4	+	-	-
G 13	-	-	-		S9	-	-	-
G 16	-	-	-		H2	+	-	-
G 18	-	-	-		H4	-	-	-
G 19	-	-	-		H9	+	-	+
G 20	-	-	-		P1	-	-	-
G 22	-	-	-		B2	+	-	-
G 23	-	-	-		M3	++	-	-
G 25	-	-	-		C2	+	-	-
G 26	-	-	-		C4	-	+	-
G 27	-	-	-		C5	+	-	-
G 28	-	-	-		C7	-	-	-
G 29	-	-	-		G2	-	-	-
G 30	++	-	-		G6	-	-	-
G 31	-	-	-		G8	-	-	-
G 32	++	++	-		G7	+	+	-
G 35	-	++	-		G1	-	-	-
G 36	-	++	-		G9	-	-	-
G 38	-	-	-		G 46	-	-	-
G 44	+	-	-		*Pf* CHA0	+++	-	-

2.3.9 Antibacterial and antifungal activity by fluorescent pseudomonad:

Fluorescent *Pseudomonas* strains were checked for their activity against *R.bataticola* and *Staphylococcus aureus* for the bioassay for 2, 4-DAPG production (Table 2.10 and Fig. 2.16).



(a) (b) (c) (d)

Fig. 2.16 Bioassay using *R. bataticola* and *S. aureus* by fluorescent *Pseudomonas* isolates. (a) extract applied to *S. aureus* (b) extract applied to *R.bataticola* (c) *Pf* CHA0 extract applied to to *S.aureus* and (d) Negative control

Table 2.10: Antifungal and antibacterial activity of isolates against *R. bataticola* and *S. aureus* deaminase activity by fluorescent pseudomonad isolates

Rhizospheric isolates	Antibacterial activity	Antifungal activity
Pf CHA0	+++	+++
G1	++++	+++
C7	+++	++++
G3	++++	++
G2	+++++	++++
G8	+++	++++
C2	+++	++++
G4	+	+++
G9	+	++
H9	++	+++
H4	++	+++
G7	+	++
G6	+	++
S4	+	+++
C5	+	++++
M3	+	+++
P1	++	++
G5	+	++

2.3.10 Characterization of PGPR strains for the presence of ACC/ KMBA pathway

2.3.10.1 ACC as a Nitrogen source

ACC and NH_4Cl both act as a nitrogen sources for PGPR strains (Blaha et al., 2006). Table 2.11 depicts strains utilizing either of the two nitrogen source. Strains G35, G19, P33, P36, P35 exhibited dense growth on plate containing ACC and G29, G1, G8 show moderate growth and standard strain *Pf*-5 along with G14, G13, G46, H9 were weak ACC utilizers. G29 strain showed the highest ACC deaminase activity while standard strain Pf-5 exhibited low ACC deaminase activity (Fig.2.17).

Table 2.11 Growth of PGPR strains on the medium containing ACC or NH_4Cl as a sole nitrogen source.

Strong ACC utilizers			
Strains	ACC as a N source	NH_4Cl as a N source	Without N source
G35	+++	+++	-
G19	+++	+++	-
Moderate ACC utilizers			
G29	++	+++	-
G1	++	+	-
G8	++	++	-
Weak ACC utilizers			
Pf-5	+	+	-
G14	+	+	-
G13	+	++	-
G46	+	+	-
H9	+	+	-

2.3.10.2 KMBA pathway in fluorescent pseudomonad strains:

Pf5, G45, G2, H4 were giving yellow precipitates when 2, 4-dinitrophenyl hydrazine (2, 4- D) was added as depicted in fig. 2.18, while un- inoculated medium did not give precipitate on addition of 2, 4-D, indicating that isolates Pf5, H9, G29, G13, G35, H4 have mechanism of utilizing ACC as nitrogen source to decrease ethylene production while others utilized KMBA

pathway (Table 2.12). Precipitates are obtained in strains having KMBA pathway functional, which is the intermediate product in ethylene biosynthesis via L- methionine. L- Met is converted to C_2H_4 by the transaminase pathway. Strains H9, G29, G13, G35, and H4 utilized both ACC and KMBA pathway (Table 2.12).

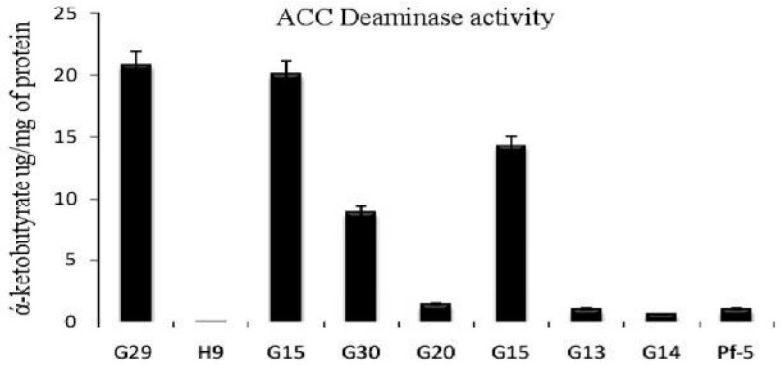
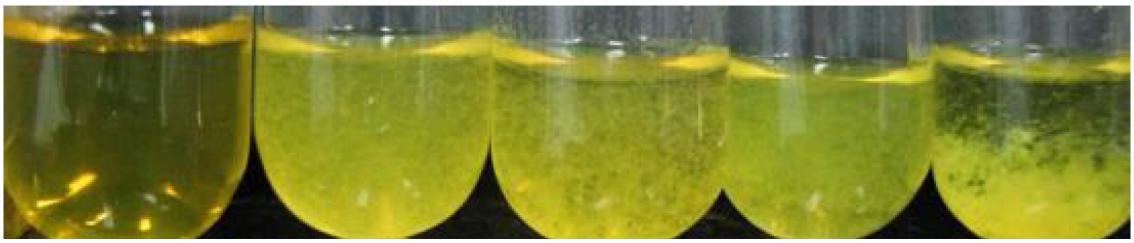


Fig. 2.17 ACC deaminase activity of representative fluorescent *Pseudomonas* strains. (Bars indicate S.D., n=3)

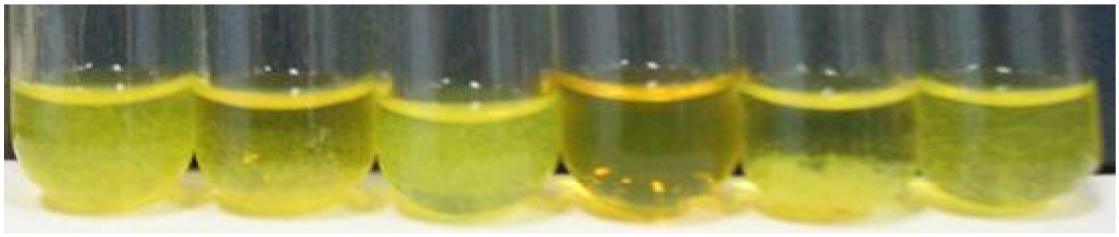
Table 2.12: Growth of fluorescent *Pseudomonas* on medium containing ACC /L-Methionine

Strains	ACC	L-Methionine
Pf-5	+	+
H9	+	+
G29	++	+
G13	+	+
G35	+	+
H4	+	+
C7	-	+
G8	-	+
CHA0	-	+
C2	-	+
G45	-	+
G16	-	+
G18	-	+
G38	-	+

Strains	ACC	L-Methionine
G2	-	+
G22	-	+
G5	-	+
G31	-	+
G7	-	+



Un-inoculated Pf-5 G45 G2 G22



G38 G31 C7 Un-inoculated G29 G7

G29 G18 G22 Uninoculated G5 G38

Fig. 2.18 Precipitation formation assay for KMBA pathway in fluorescent pseudomonad

2.3.11 HCN production by fluorescent *Pseudomonas* isolates:

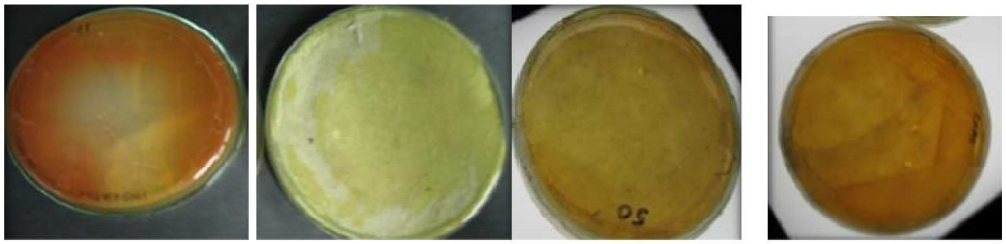
Many *P. fluorescens* strains have been reported to produce HCN and this contributes to the biocontrol activity of the strain. Isolates like G19, G20, G25, G30, G31 and G35 have shown less intense brown colour, while isolates like G2, G8, C7, G1, G44 and G45 have shown brown

colour equally intense as that of *Pf* CHA0 (Fig. 2.19 and Table 2.13), while rest of the isolates did not show change of colour, thus they are considered as HCN negative strains.

Table 2.13 HCN production by fluorescent *Pseudomonas* isolates

Strains	HCN Production		Strains	HCN Production
G 8	-		T8	-
G 12	-		S4	++
G 13	-		S9	-
G 16	-		H2	+
G 18	-		H4	+
G 19	+		H9	+
G 20	+		P1	-
G 22	-		B2	+
G 23	-		M3	+
G 25	+		C2	-
G 26	-		C4	+
G 27	-		C5	-
G 28	-		C7	++++
G 29	-		G2	++++
G 30	+		G6	++
G 31	+		G8	++++
G 32	-		G7	++
G 35	+		G1	++++
G 36	-		G9	+++
G 38	-		G 45	+++
G 44	+++		G 46	-
Pf CHA0	+++			

(C7) (G2) (C2) (G8)



(G1) (Uninoculated) (G23) (G6)

Fig. 2.19 HCN production by fluorescent *Pseudomonas* strains

2.3.12 ITS-RFLP of fluorescent pseudomonas strains:

The fluorescent *Pseudomonas* strains repeatedly produced a single band of 580 bp. All genotypes showed a single PCR-amplified ITS product (580 bp). When the ITS-PCR products were digested with *Hae*III and *Msp*I, *Hae* III was found to be effective cutter and gave distinct bands. The sum of the sizes of restriction fragments agreed with sizes of undigested ITS amplification product. The *Hae*III restriction digestion patterns of the amplified rDNA ITS regions were used for cluster analysis (Fig. 2.20). Fragments of over 100 bp were included for calculating similarity matrices. The cluster analysis has revealed 13 ITS-RFLP types (Fig. 2.21). All the fluorescent *Pseudomonas* genotypes clustered with the three *Pseudomonas* reference strains. There are two major clusters in the present dendogram, one group representing the standard strain *Pf* CHA0 and its closely affiliated strains such as G36, G29 and P1 while other group is represented by standard strains Pf-5 and closely affiliated strains such as G5, G30 (Fig. 2.21).

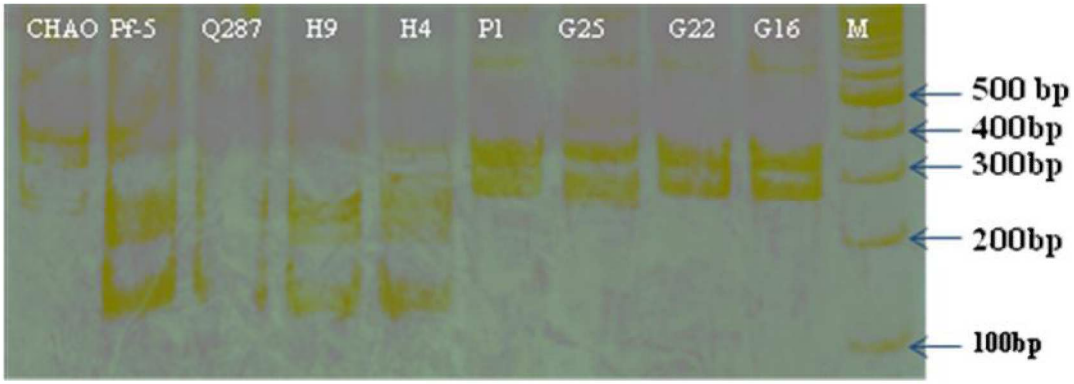


Fig. 2.20 (A)

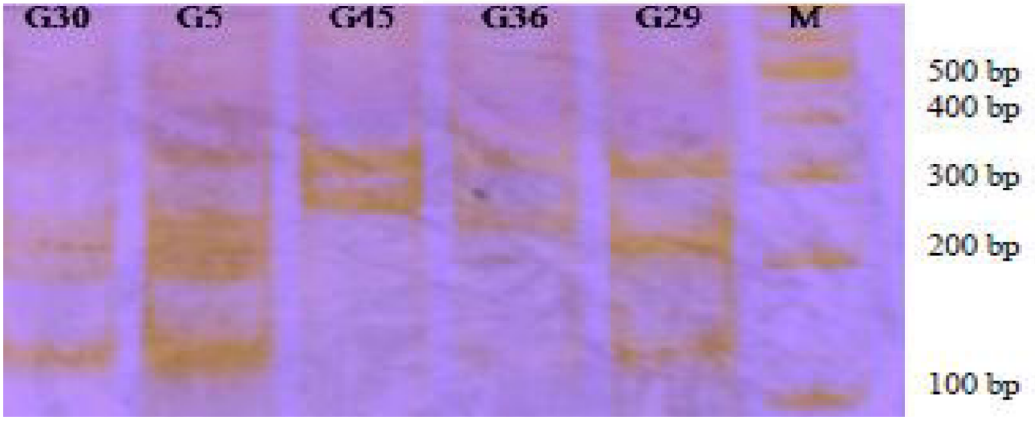


Fig. 2.20 (B)

Fig. 2.20 ITS-RFLP patterns of fluorescent *Pseudomonas* strains using *Hae III*

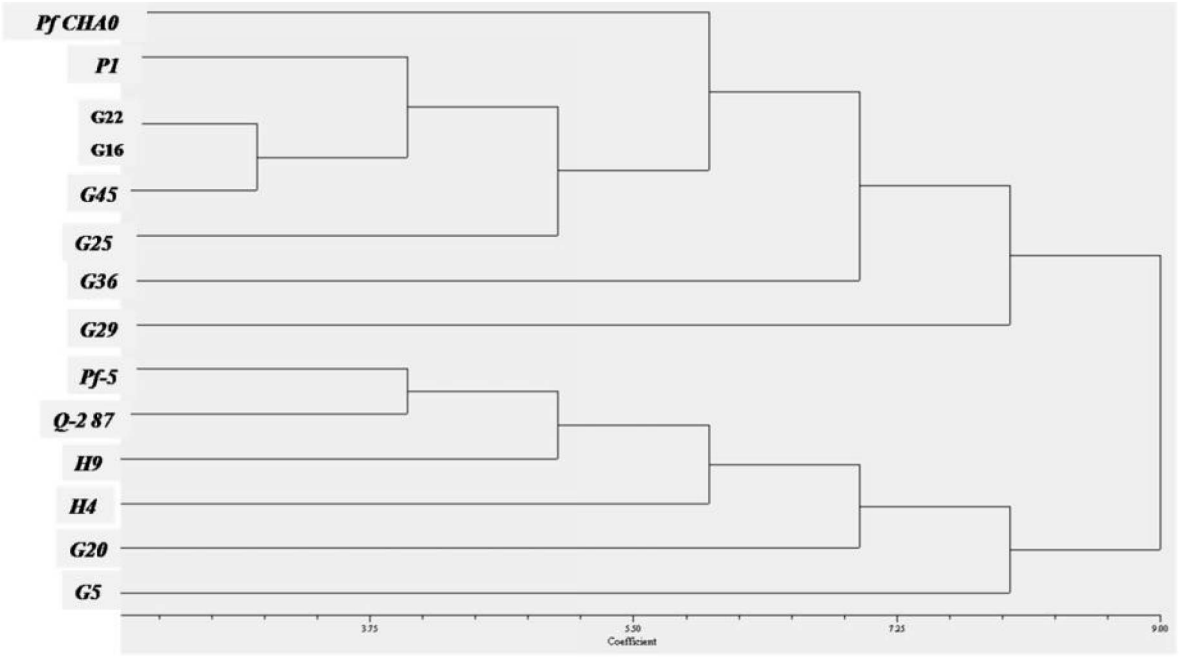


Fig. 2.21 Dendrogram of fluorescent *Pseudomonas* strains based on ITS-RFLP

2.4 CONCLUSION:

Eighty three mung bean root-colonizing fluorescent strains were isolated after using the isolation strategy for the efficient root colonizers, which was done after three rounds of plant inoculations. Isolates were checked for antifungal activity by using *R.bataticola* as an indicator fungus, which is a known pathogen for several leguminous plants including *Vigna radiata*. The strains were checked for several PGPR traits. Of these, 62 isolates were identified as fluorescent pseudomonads using specific biochemical tests. Fluorescent isolates were further confirmed as *Pseudomonas* spp. by specific ITS-PCR and 41 strains were confirmed. Diversity index of fluorescent pseudomonas was high in pigeon pea (0.5) and tobacco rhizospheres (0.269) and a number of fluorescent pseudomonad were obtained from tobacco, rice, and sugarcane and groundnut rhizosphere. ITS-RFLP dendogram revealed 13 ITS-RFLP types in isolated fluorescent *Pseudomonas* collection. Table 2.14 summarizes the PGPR traits found in the selected fluorescent PGPR strains and shows that these strains have good potential at plant growth promotion and biocontrol. The isolation and screening strategy was specific for colonization of mung bean plants and having antifungal activity against *R. bataticola; w*hether these strains have broad spectrum of colonization and antagonsism remains to be seen.

Table 2.14 Summary of PGPR traits for the fluorescent pseudomonas strains:

	Antifungal activity	P-solubilisation ability	Siderophore production	IAA production	HCN production	ACC deaminase	KMBA utilization
G35	+	+	+	+	+	+	+
G1	+	+	+	+	+	+	+
G2	+	+	+	+	+	-	+
G31	+	+	+	+	+	-	+
G45	+	+	+	+	+	-	+
G8	+	+	+	+	+	-	+
G29	+	+	+	+	-	+	-
G46	+	+	+	+	-	+	-
G6	+	+	+	+	-		
G19	+	+	+	+	+	-	+
G18	+	+	+	+	-	-	+
G44	+	+	+	+	+	-	+
H2	+	+	+	+	-	-	+
T8	+	+	+	+	-	-	+
C2	+	+	+	+	+	-	+
C5	+	+	+	+	-	-	+
C7	+	+	+	+	-	-	+

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