CHAPTER 4
Isolation and Characterization of
Pseudomonas fluorescens
4.1 Introduction

Studies on the mechanisms of biological control by PGPR (Plant growth promoting rhizobacteria) have concentrated on how PGPR antagonize pathogens (Whipps, 2001; Antoun and Kleopper, 2001). Suppression of root disease by PGPR is attributed to its ability to colonize the host root, compete for space and nutrition and inhibit the growth of the fungus by producing antibiotics or siderophores.

The advantage for the selection of potential plant growth promoting and bioprotecting rhizobacteria is very important because it is based on the different traits of PGPR. The PGPR benefit plants through different mechanisms of action, including, (i) The production of secondary metabolites such as antibiotics, cyanide, and hormone like substances; (ii) The production of siderophores; (iii) Antagonism to soilborne root pathogens; (iv) Phosphate solubilization; and (v) Dinitrogen fixation (Bakker and Schippers, 1987; Dart, 1986; Dubeikovsky 1993; Goldstein 1986; Leong, 1986; Weller, 1988). The establishment in the rhizosphere of organisms possessing one or more of these characteristics is interesting since it may influence the growth of plant. Bakker et al. (2003), Compant et al. (2005) and Podile and Kishore (2006) reviewed the different mechanism of plant growth promotion and disease protection which includes, production of phytohormones, ACC deaminase, Phosphate solubilization, Siderophore, antibiotic production, Competition, lytic enzymes etc. One of the most important mechanisms responsible for the suppression of plant pathogens for Pseudomonas spp. is siderophore-mediated competitions for iron (Henry et al., 1991). Pseudomonas spp. are often used as model root-colonizing bacteria (Lugtenberg et al., 2001). The beneficial effects of these rhizobacteria have been variously attributed to their ability to produce various compounds including phytohormones, organic acids, siderophores, fixation of atmospheric nitrogen, phosphate solubilization, antibiotics and some other unidentified mechanisms (Glick, 1995).

The selection of bioantagonistic rhizobacteria, in order to take into account the direct effect on pathogen development, must consider conditions where the bioantagonist should develop, i.e. salinity and pH of soils and different temperature, among others. Therefore objective of this work is the isolation of antagonistic P. fluorescens that could control Fusarium oxysporum f.sp. lycopersici, in in vitro and in vivo and their characterization in terms of
morphological, physiological, biochemical and antagonistic mechanisms used to control the pathogen and conditions for growth similar to those present in the field.

Objectives of the study

- Isolation and selection
- Characterization of rhizobacteria
  a. Morphological characterization
  b. Physiological characterization
  c. Biochemical characterization
  d. Molecular identification
  e. Characterization for their PGPR traits

4.2 Materials and methods

4.2.1 Isolation and selection of rhizobacteria

In order to isolate rhizobacteria from the rhizosphere soil sample, 10 g of soil were suspended in 100 ml of saline (0.85% NaCl), shaken for 30 min, and the soil suspensions were then serially diluted (8-fold) and plated onto Luria bertain (LB) agar medium in petri dishes which were incubated at 28±2°C (Silva et al., 2003). At the end of 36-48 h incubation individual colonies which differ in their morphological characters such as, colony character, were transferred to LB agar slants. To avoid other bacteria from bulk soil, the bacteria were isolated whose population was more than 10% of total bacterial population of the particular rhizosphere soil sample.

All the rhizobacterial isolates were maintained on LB agar slants at 4°C for short term storage and for long term storage, the bacteria was stored in 40% glycerol at -80°C.

4.2.1 Dual culture

The bacterial isolates were screened for their antagonistic activity against the *F. oxysporum* f.sp. *lycopersici* by employing the dual culture technique. The interaction was studied in 90-mm diameter petriplate containing PDA. Four isolates were point inoculated on each plate. Plates were incubated for 2 days at 28±2 °C, and one 5-mm diameter agar disc of *F. oxysporum* f.sp.
lycopersici from a one week old PDA culture was placed in the centre of the plate. After 7 days of incubation, the width of the inhibition zone was measured for each *P. fluorescens* isolate and the inhibition was calculated according to the formula given by Ahmed *et al.* (1999).

\[ I=100-(100 \frac{R_2}{R_1}) \]

Where I is the degree inhibition of vegetative growth of the fungi, \( R_1 \) is the radius of the control colony in mm, and \( R_2 \) is the distance traveled by the *F. oxysporum* f.sp. *lycopersici* colony. The experiment was conducted in triplicate and was repeated twice. Student test was performed as \( P \leq 0.05 \).

### 4.2.3 Characterization of rhizobacteria

#### 4.2.3.1 Morphological characterization

The colony morphology was studied by inoculating the test isolates onto KB (King’s B medium), YDC (Yeast Dextrose Calcium carbonate agar), NBY (Nutrient Broth Yeast Extract), GF (Growth Factor) and NA (Nutrient Agar) media. Bacterial colonies were observed for the shape, size, margin *etc.*

#### 4.2.3.2 Physiological characterization

Physiological characterization of rhizobacteria was studied by undertaking the following tests.

##### 4.2.3.2.1 Effect of different pH values on development of antagonistic bacteria

An aliquot of 0.5mL of a rhizobacterial suspension (5×10^9 cfu mL\(^{-1}\)) from a 24 hrs old culture was placed in a tube containing 4.5mL of peptone broth previously adjusted at the desired pH values which ranged between 5.0 and 8.0 (with intervals each 1 pH units). Tubes were incubated for 48hrs at the temperatures mentioned above, and bacterial growth was estimated at 600nm, after serial dilutions when necessary. The experimental unit was one tube for each experimental condition. Results correspond to the mean of all experiments, which were repeated at least three times.
4.2.3.2.2 Effect of different temperatures on development of antagonistic bacteria

An aliquot of 0.5mL of a rhizobacterial suspension (5×10⁹ cfu mL⁻¹) from a 24 hrs old culture were placed in a tube containing 4.5mL of peptone broth and kept under different temperatures of incubation of 5,10,15,28 and 37°C. Tubes were incubated for 48 hrs at the temperatures mentioned above, and bacterial growth was estimated at 600nm, after serial dilutions when necessary. The experimental unit was one tube for each experimental condition. Results correspond to the mean of all experiments, which were repeated at least three times.

4.2.3.2.3 Effect of different salt concentrations on development of antagonistic bacteria

The growth of rhizobacterial isolates were tested under NaCl concentrations of 0, 50,100,150,200 and 250mM. An aliquot of 0.5mL of a bacterial suspension (1×10⁸ cfu mL⁻¹) from a 24 hrs old culture were placed in a tube containing 4.5mL of peptone broth previously adjusted at the desired NaCl concentration. Tubes were incubated for 48 hrs at the temperatures mentioned above, and bacterial growth was estimated at 600nm, after serial dilutions when necessary. The experimental unit was one tube for each experimental condition. Results correspond to the mean of all experiments, which were repeated at least three times.

4.2.3.2.4 Effect of Fe³⁺ on development of antagonistic bacteria

This effect was tested according to Pumarino, 1995 using FeCl₃·6H₂O at 0, 5, 10, 15 and 20 ppm added to the King B medium. An aliquot of 0.5mL of a bacterial suspension (1×10⁸ cfu mL⁻¹) from a 24 hrs old culture were placed in a tube containing 4.5mL of King’s B broth previously adjusted at the desired FeCl₃ concentration. Tubes were incubated for 48 hrs at the temperatures mentioned above, and bacterial growth was estimated at 600nm, after serial dilutions when necessary. The experimental unit was one tube for each experimental condition. Results correspond to the mean of all experiments, which were repeated at least three times.

4.2.3.3 Biochemical characterization

Biochemical tests for identification were done following the Bergey’s manual of determinative bacteriology (1994) and Cuppuccino and Sherman (2004). Isolated bacteria were sub cultured on to NA (Nutrient agar) medium and 24-36-h-old-cultures were used and each test
was conducted with four replicates and repeated twice for all biochemical tests. The biochemical tests were carried out with appropriate controls following the standard procedures.

4.2.3.3.1 Gram’s reaction

This test is essential to differentiate bacteria into gram positive and gram negative bacteria. A loopful of bacterial suspension was smeared on to a glass slide. It is air-dried and heat fixed by passing the slide rapidly two to three times on Bunsen burner. The smear was flooded with crystal violet solution for 1 min. The slide was washed with a gentle stream of tap water blot dried and flooded with lugol’s iodine for 1 min. Again the slide was washed with water and blot dried, and decolorized by washing in a gentle stream of 95% ethyl alcohol for 30 sec to remove excess stain that will easily wash away, counter staining was done by flooding with safranin for 20 sec. The slide was again washed with tap water and blot dried. The preparation was observed under compound microscope at different magnifications for pink-red or blue-violet stained bacteria representing gram-negative or gram-positive nature respectively.

4.2.3.3.2 KOH solubility test

The main principle behind this test is that, the lipopolysaccharides present in the bacterial cell wall gets dissolved in 3% KOH and forms a mucoid thread. A loopful of bacteria from a well grown colony was mixed in a drop of 3% aqueous KOH solution for not more than 10 seconds with the help of a toothpick. Tooth pick was raised a few centimeters from the microslide and was observed for the formation of a mucoid thread. The gram positive bacteria do not produce strands even on repeated strokes of the toothpick.

4.2.3.3.3 Catalase test

24-h-old-slant-culture of the test bacterium was flooded with one ml of 3% hydrogen peroxide and observed for the production of gas bubbles.

4.2.3.3.4 Kovac’s oxidase test

The principle behind Kovac’s oxidase test is that, those bacteria which can produce oxidase enzyme can oxidize 1% aqueous solution of Tetra methylele-para-phenylene diamine dihydro chloride.
A loopful bacterial culture was rubbed on a disc of Whatmann filter paper No. 1 impregnated with a freshly prepared 1% (w/v)solution of tetra methyl-p-phenylene diamine dihydro chloride in distilled water and observed for the reagent to a purple colour within 10 sec of application of the culture (Kovac, 1956).

4.2.3.3.5 Starch hydrolysis

Starch is an insoluble polymer of glucose. Some bacteria possess the ability to produce amylase that breaks starch into glucose units and amylase an extra cellular enzyme which is released by microorganisms. Starch agar was prepared, sterilized and dispensed into sterile petriplates. The plates were inoculated with the test isolates. After incubation at 37 °C for three days, the culture plates were flooded with Lugol’s Iodine and the plates were observed for the formation of a clear zone around the bacterial growth.

4.2.3.3.6 Lipase activity

This test is used to determine whether an organism can produce lipase enzyme or not. Lipase enzyme hydrolyzes the Tween 80 into free fatty acids. Tween 80 agar medium was autoclaved and poured into sterile petriplates. The plates were streak inoculated with test isolates. The plates were incubated for three days at 37°C and observed for the development of a milky precipitate around the colony. Ten isolates are positive for the lipase test if white granular precipitate is seen around the colonies.

4.2.3.3.7 Casein hydrolysis

This test is used to determine whether the bacteria can hydrolyze casein by the action of enzyme casein hydrolase. Double strength nutrient agar medium and skimmed milk powder solution (10%) was sterilized in two different flasks. Both were mixed well before pouring into sterile petriplates. The plates were streak inoculated with the test isolate and incubated at 37°C for 48 h. Bacterial cultures were recorded positive if zone of hydrolysis was seen around the colonies.

4.2.3.3.8 Oxidation/fermentation test:
The oxidation/fermentation semi solid media was prepared and dispensed in equal volumes to test tubes and sterilized. To the cooled medium separately autoclaved 10% w/v glucose solution was added to each of the tubes to obtain final concentration of 1%. Test tubes were stab inoculated in duplicates and one set of the tubes were sealed with sterile liquid paraffin to create an anaerobic condition. Uninoculated sealed and open tubes served as control. The tubes were incubated at 30 °C in the BOD incubator and observed for the change of colour from green to yellow (Fahy and Persley, 1983).

4.2.3.3.9 Gelatin hydrolysis

This test is used to determine whether an organism can hydrolyze gelatin by the action of gelatinase enzyme. Test tubes containing gelatin medium was stab inoculated. Uninoculated control and inoculated tubes were incubated at 37°C for 2-3 days.

4.2.3.3.10 Action on litmus milk

Since milk is usually the first substrate used to maintain bacteria, this test allows for accurate depiction of bacterial types. The addition of litmus, other than explaining the pH type, acts as an oxidation-reduction indicator. 5 ml of 4% aqueous commercial litmus milk (Difco) solution was dispensed into test tubes and autoclaved at 121°C for 20 min. After cooling, the tubes were inoculated with test isolates. An uninoculated tube served as negative control. The tubes were incubated at 37°C for three days and change in the colour of litmus milk was observed. A change from purple to pink indicates alkaline reaction and change of colour from violet to pink indicates acidic reaction.

4.2.3.3.11 Nitrate reduction test

The nitrate test reduction is to differentiate between bacteria based on their ability to or inability to reduce nitrate (NO₃⁻) to nitrate (NO₂⁻) using anaerobic respiration. Nitrate semi-solid medium is prepared and distributed into tubes at the rate of 5ml per tube. The sterilized cooled tubes were inoculated with the bacterial isolates along with the uninoculated control and incubated at 37°C for 2-3 days. Shaking of the tubes was avoided to prevent dissolution of oxygen, which inhibits the reaction. After the incubation period, three-four drops of solution one and solution two was added and observed for black or blue colour development.
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Solution 1 was prepared by mixing solution A (A=starch=0.4g/100 ml and Zinc chloride was dissolved in 10 ml water, boiled to that starch was added and diluted to 100ml. Allowed to stand for one week and then filtered the solution) and solution B= (B=0.2% KI) in the ratio 1:1. Solution 2 was prepared by mixing 16 ml concentrated HCL and 86ml distilled water (Fashy and Persley, 1983).

4.2.3.3.12 Arginine dihydrolase test

The basic principle behind the test is ammonia is evolved under anaerobic condition, which brings about the change in pH of the media to alkaline. So the light pink coloured Thornel’s media turns to dark red indicating a positive reaction. A fresh culture tube containing 5 ml of sterilized thornel’s medium was stab inoculated with the test isolate. The surface of the medium was sealed with sterile molten Vaseline. Uninoculated tube served as negative control. The tubes were incubated at 37°C for three days and observed for the change in colour of litmus milk. Change in colour from orange to red indicates positive result.

4.2.3.3.13 Citrate utilization test

The bacterium incubated onto Simmon’s citrate agar slants, one tube kept as uninoculated control. All the slants were incubated at 37 °C for 48h. If the colour of the medium changed from green to blue then the bacterium was considered as positive for citrate utilization. Unchanged green colour of the medium indicates negative results.

4.2.3.14 Protein estimation

Protein estimation was done following the method of Lowry et al. (1951). Equal concentration of protein was taken for Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE). SDS PAGE was carried out according to the procedure of Lamelli et al., using a discontinuous buffer system.

4.2.4 Molecular confirmation of Pseudomonas fluorescens by 16S ribosomal RNA Sequencing

All selected rhizobacteria were identified up to their genus level by conducting biochemical and physiological tests according to Bergey’s Manual of Systemic Bacteriology

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(Krieg and Holt, 1984) and Microbiology – A laboratory manual (Cuppuccino and Sherman, 2004). Further the selected rhizobacterial isolates were identified by 16S rRNA sequencing and aligned with the reference 16S rRNA gene sequence using the BLAST algorithm according to the method of Altschul et al. (1997).

4.2.5 Characterization of selected rhizobacteria for their PGPR traits

4.2.5.1 Root colonization bioassay

This bioassay was performed as explained according to the procedure of Silva et al. (2003). Surface sterilized tomato seeds were soaked in 25 ml of *P. fluorescens* suspension of (1×10^8 cfu mL^-1) for 24 h and then transferred to sterile 0.6 percent water agar tubes. The seedlings were allowed to grow at room temperature. Periodic visual inspections were performed daily in order to detect bacterial growth around arising roots.

4.2.5.2 Indole acetic acid (IAA)

Production of indoles by rhizobacteria was assayed as described by Patten and Glick (1996). Bacterial isolates were propagated in LB broth (1/10 strength) with L-tryptophan (500 μg/ml) and incubated at 28±2°C for 48 h. Bacterial cells were removed by centrifugation at 8,000 rpm for 10 min at 4°C. One ml of the supernatant was mixed with 4 ml of Salkowski’s reagent in the ratio of 1:4 and incubated at room temperature for 20 min. Development of a pink colour indicated indoles.

4.2.5.3 Phosphate solubilization

Ability of isolated rhizobacteria to solubilize phosphate was determined on Pikovskaya’s medium (Pikovskaya, 1948). Plates inoculated with rhizobacteria were incubated at 28±2°C, analyzed for clear zone around the bacterial colony up to 10 days. The bacterium which showed zone of clearance on repeated subculture onto Pikovskaya’s medium was considered as positive for phosphate solubilization.

4.2.5.4 Siderophore

Siderophore production was tested qualitatively using chrome azurol S (CAS) agar as described by (Schwyn and Neilands, 1987; Alexander and Zuberer, 1991). The bacterial culture
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was spotted on the CAS agar plates with two replications. Orange halos around the colonies after 1-5 days of incubation indicated siderophore production.

4.2.5.5 Chitinase

Ability of the rhizobacterial isolate to produce chitinase was determined by inoculating on minimal medium amended with colloidal chitin as the sole carbon source (Renwick et al., 1991). Plates were incubated at 28±2°C, analyzed for zone of clearance up to 10 days. The bacterium which showed distinct zone of clearance on repeated subculture was considered as positive for chitinase production.

4.2.5.6 Cyanide production

Cyanide production was determined in slants of 0.1 x NA amended with Glycine (4.4 g/l) and FeCl₃.6H₂O (0.3mM) (Castric, 1977). A strip of sterilized filter paper saturated with a solution containing picric acid, 0.5 % and Sodium carbonate (2.0%) was placed inside the slants which were already inoculated with test bacteria, such that the filter paper doesn’t touch the medium and tubes were sealed air tightly (Bakker and Schippers, 1987). The slants were incubated for 4-7 days at room temperature. Change in colour of the filter paper from yellow to brown was considered as positive reaction.

4.2.5.7 Cellulase

Production of cellulase was determined on basal medium supplemented with Carboxymethyl cellulose (CMC) (10 g⁻¹) (Cattelan et al., 1999).

Statistical analysis: All data from laboratory and greenhouse experiments were analyzed separately for each experiment and were subjected to arcsine transformation and analysis of variance (ANOVA) (SPSS, version 16). Significant effects of treatments were determined by the F values (P≤0.05). Treatment means were separated using Tukey’s HSD test.

4.3 Results

4.3.1 Direct antagonism through Dual cultures

In dual culture test not all the isolates inhibited the growth of F. oxysporum. Among all isolates (Isolates 1-20), isolates 1, 5 and 18 showed maximum inhibition of mycelial growth. Among them isolate1 resulted in 65% inhibition was found to be most effective isolate in
suppressing the growth of *F. oxysporum* compared to other isolates. Isolate 5 and 18 also inhibited the growth of *F. oxysporum* 60 and 55% respectively. Control plates not treated with the bacterial isolates were completely covered by the *F. oxysporum* showing no inhibition. The mean mycelial growth inhibition of the most effective bacterial isolates revealed that the inhibition was highly significant (*P*=0.05). The three isolates (isolates 1, 5 and 18) which showed maximum inhibition of mycelia growth were selected for further study (Table 4.1, Fig 4.1).

4.3.2 Morphological characterization

The Morphological characters of the three rhizobacterial isolates on NBY were white precipitate colonies. Hollow transparent colonies were observed on YDC medium. However, the growth of rhizobacteria was luxuriant on KB medium. The 3 rhizobacteria consisted of fluorescent bacteria as determined on King’s medium B under UV\textsubscript{366 nm} radiation (Fig 4.1).

4.3.3 Physiological characterization

4.3.3.1 Rhizobacterial growth at different pH values

Isolates 1, 5 and 18 grew best at pH values 5, 6 and 7 and showed its highest growth at pH 6 and 7. The optimum pH was determined in a range 5-7 (Fig. 4.2).

4.3.3.2 Rhizobacterial growth at different temperatures

The poor growth of all the three bacteria was observed at 5 °C and 10 °C and little growth observed at 15 °C and better growth at 28 °C and no growth at 45 °C. All the isolates grew well at 37°C. Therefore optimum temperature for the growth of all the three isolates was 28-37 °C (Fig.4.2).

4.3.3.3 Effect of different NaCl concentrations on the growth of rhizobacterial isolates

The growth of all the three bacteria was tested under different concentrations of NaCl. The best growth of all the three bacterial isolates was observed at 50, 100, 150 and 200mM NaCl. Less growth was observed at 250mM NaCl concentration inhibited. Concentrations of 250mM NaCl inhibited the growth (Fig. 4.2).
4.3.3.4 Effect of Fe$^{3+}$ on the growth of rhizobacterial isolates

The three antagonistic bacteria showed similar behaviour on the growth of rhizobacteria at the Fe$^{3+}$ concentrations tested. All the bacteria showed better growth at iron concentration at 50ppm. Therefore iron concentration less than 50 ppm induce siderophore production (Fig. 4.2).

4.3.4 Biochemical Characterization of rhizobacterial isolates

4.3.4.1 Gram staining

The test isolates stained red indicating that bacteria are gram negative. They appeared as rods Known gram positive bacteria showed violet colour, which was compared with our test isolates (Fig. 4.3).

4.3.4.2 KOH solubility test

The test isolates produced thin viscidmucoid strand when a loopful of test bacteria was mixed with 3% KOH solution (Fig. 4.3).

4.3.4.3 Catalase test

Bubbles were formed when the slant cultures of the test isolates were flooded with 3% H$_2$O$_2$ indicating the test isolates as catalase positive (Fig. 4.3).

4.3.4.4 Kovac’s oxidase test:

The test isolates gave a positive reaction for Kovac’s oxidase test. The whatmann paper turned bluish purpleat the site of inoculation even after ten seconds of rubbing on the filter paper impregnated with Kovac’s oxidase reagent (Fig. 4.3).
4.3.4.5 Starch hydrolysis:

The test isolates failed to produce a clear zone of hydrolysis when the culture plates were flooded with Lugol’s iodine. A clear zone of hydrolysis was formed around the positive control. Hence the test was negative for starch hydrolysis (Fig. 4.3).

4.3.4.6 Lipase activity

The formation of white precipitate around the colonies of test isolates was absent indicating that Tween 80 was not hydrolyzed. Hence the bacterium is lipase negative (Fig. 4.3).

4.3.4.7 Casein hydrolysis:

Bacterial cultures were recorded positive, zone of hydrolysis was seen around the colonies (Fig. 4.3).

4.3.4.8 Oxidation/fermentation

In the oxidation test there was change of colour from green to yellow but in fermentation test there was no change in colour from green to yellow. Acid without gas is produced aerobically in the medium containing glucose (Fig. 4.4).

4.3.4.9 Gelatin hydrolysis

The test isolates were recorded as negative, as liquefaction of the gelatin medium was not observed after incubation. Xanthomonas oryzae was used as positive control for the test which showed liquefaction of the test (Fig. 4.4).

4.3.4.10 Action on litmus milk

Change in colour of the litmus milk from purple to pink was not observed in tubes inoculated with test isolates after three days of inoculation (Fig. 4.4).

4.3.4.11 Nitrate reduction

The result for nitrate reduction was positive for test isolates. The medium turned black on addition of solutions 1 and 2 (Fig.4.3).
4.3.4.12 Arginine dihydrolase

The bacteria recorded positive for arginine dihydrolase activity. There was change in colour from pink to red (Fig. 4.4).

4.3.4.13 Citrate utilization

The colour of the medium changed from green to blue. The bacterium was considered as positive for citrate utilization (Fig. 4.3)

4.3.14 Protein estimation

Different bands of intra molecular protein were seen in three rhizobacterial isolates 1, 5 & 18 and they were compared with the Standard molecular weight protein. All the molecular weight bands come in between molecular weights of 14.3 kDa and 97.4 kDa of Standard molecular weight proteins. Based on morphological, physiological and biochemical tests the rhizobacteria were identified as *Pseudomonas fluorescens* and renamed all the rhizobacterial isolates as Pf1, Pf2, Pf3, Pf4, Pf5, Pf6, Pf7, Pf8, Pf9, Pf10, Pf11, Pf12, Pf13, Pf14, Pf15, Pf16, Pf17, Pf18, Pf19 & Pf20. Further confirmation was done by Molecular identification.

4.3.4 Molecular confirmation of *Pseudomonas fluorescens* by 16S ribosomal RNA:

The identification of the *Pseudomonas fluorescens* isolates was further confirmed by molecular identification. Identification of *P. fluorescens* isolates were confirmed by using polymerase chain reaction (PCR) by 16S ribosomal RNA amplification and it was cloned into and sequenced. The 16S ribosomal RNA gene was sequenced and aligned with the reference 16S ribosomal RNA gene sequence using the BLAST algorithm.

The 611 base pair amplicon was cloned into TOPO PCR4 (Invitrogen) and sequenced. Using the BLAST algorithm for analysis, the sequence showed 98% to 99% identity to several isolates of *P. fluorescens*. Among 20 isolates which were characterized as *P. fluorescens*, 12 isolates were identified as *P. fluorescens* (Pf1, Pf2, Pf3, Pf4, Pf5, Pf9, Pf10, Pf13, Pf14, Pf17, Pf18 and Pf19) (ACCESSION: HQ202899; HQ202900; HQ202901; HQ202902; HQ202903; HQ202904; HQ202905; HQ202906; HQ202907; HQ202908; HQ202909; HQ202910) by 16S ribosomal RNA gene sequencing.
Therefore the three *P. fluorescens* isolates (Pf1, Pf5 & Pf18), identified and confirmed as *P. fluorescens*, were further studied for their induction of systemic resistance (ISR) and their effect on plant growth, yield and disease suppression under greenhouse and field conditions in Objective III and IV.

### 4.3.5 Characterization of selected rhizobacteria for their PGPR traits

All the three rhizobacteria were positive for root colonization, siderophore production, cellulase, chitinase, IAA production, cyanide production and phosphate solubilization (Fig 4.5, Table 4.3.)
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Figure 4.1. (i) a. Isolation of *Pseudomonas fluorescens* on King’s B media, b. Screening for bioantagonistic activity by dual culture (Rb-Rhizobacetrria and *Fusarium oxysporum*).

(ii) Morphology study on of rhizobacterial isolates on different media like, (c) King’ B, (d) Growth factor, (e) Yeast dextrose calcium carbonate agar, (f) Nutrient agar, (g) Nutrient broth yeast dextrose agar, (h) Tween 80.
Figure. 4.2: Influence of temperature, pH, NaCl concentration, Ferric chloride on the growth of rhizobacterial isolates. The data expressed as the average of three independent experiments with three replicates each. IS1=Isolate 1, IS5=Isolate 5, IS18=Isolate 18.
Figure 4.3. Biochemical characterization of rhizobacteria: (a & b) Gram staining, (c) KOH solubility test, (d) Catalase test, (e) Kovac’s oxidase test, (f) Starch hydrolysis test, (g) Lipase activity test, (h) Casein hydrolysis.
Figure 4.4. Biochemical characterization. (a) Oxidation/Fermentation, (b) Gelatin hydrolysis, (c) Litmus milk test, (d) Nitrate reduction, (e) Arginine dihydrolase test, (f) Citrate utilization.
Figure: 4.5. Characterization of selected *Pseudomonas fluorescens* isolates for their PGPR traits: (a) Root colonization, (b) Indole acetic acid production, (c) Hydrogen cyanide production, (d) Phosphate solubilization, (e) Cellulase test, (f) Siderophore test, (g) Chitinase test.
Figure 4.6 SDS PAGE: Different bands of protein seen in
a. Standard molecular weight protein, b. Pf1, c. Pf5, d. Pf18
Table 4.1 *In vitro* antagonism assay of rhizobacterial isolates against *F. oxysporum* f.sp. *lycopersici*.

<table>
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<tr>
<th>Sl.No.</th>
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<th><em>P. fluorescens</em> inhibition zone (cm)</th>
<th>% Inhibition</th>
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<td>09</td>
<td>9</td>
<td>K.R.Pet</td>
<td>0.80±11&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>16±1.15&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Sakaleshpur</td>
<td>1±0.14&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>20±1.73&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>Bagepalli</td>
<td>1.6±0.08&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>32±2.88&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>Chintamani</td>
<td>0.8±0.11&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>20±1.43&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>Ramanagara</td>
<td>0.9±0.17&lt;sup&gt;def&lt;/sup&gt;</td>
<td>25±3.46&lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>Channapatna</td>
<td>1.2±0.14&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>30±3.46&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>Anekal</td>
<td>1.4±0.11&lt;sup&gt;bcdde&lt;/sup&gt;</td>
<td>32±2.88&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>Hassan</td>
<td>0.5±0.05&lt;sup&gt;defg&lt;/sup&gt;</td>
<td>18±1.15&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>Chikkaballapur</td>
<td>2.0±0.17&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>55±1.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>Mulabagilu</td>
<td>2.2±0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>62±4.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Nagamangala</td>
<td>0±0&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0±0&lt;sup&gt;lj&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>Arsikere</td>
<td>1.2±0.11&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>32±1.73&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Growth of bacterial isolates was evaluated after 72 hours at 22°C in PDA medium. The Experiment was performed three times and values are the mean with in the column sharing the same letters are not significantly different according to Tukey’s HSD at p≤0.05.
Table 4.2 Biochemical characterization of rhizobacteria:

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Biochemical tests</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gram’s reaction</td>
<td>Gram negative</td>
</tr>
<tr>
<td>2</td>
<td>KOH solubility test</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Kovac’s oxidase test</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Starch hydrolysis test</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Lipase activity test</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Casein hydrolysis test</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Oxidation/fermentation test</td>
<td>+/-</td>
</tr>
<tr>
<td>9</td>
<td>Gelatin hydrolysis test</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Litmus milk test</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Nitrate reduction test</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Arginine dihydrolase test</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Citrate utilization test</td>
<td>+</td>
</tr>
</tbody>
</table>

All the tests were conducted in four replicates and were repeated thrice, ‘+’ indicates positive reaction, ‘-’ indicates negative reaction.

Table 4.3: Characterization of selected rhizobacteria for their PGPR traits

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Tests for PGPR traits</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Root colonization test</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Siderophore test</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>IAA test</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Cellulase</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Chitinase</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Phosphate solubilization</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Cyanide production</td>
<td>+</td>
</tr>
</tbody>
</table>

All the tests were conducted in four replicates and were repeated thrice, ‘+’ indicates positive.
4.4 Discussion

Screening strategies call for recovery and characterization of large bacterial gene pools from various representative ecosystems in order to identify the desired properties. Within the present scenario, the most well studied phenomenon is the antagonistic activity of rhizosphere microorganisms towards plant pathogens with the resultant suppression of plant disease (Glick 1995; Emmert and Handelsman 1999; Weller et al., 2002). However, a major problem with such biological agents is inconsistency in field performance, which is not only the net outcome of complex interactions involving plant, biological agents, pathogen and the physical and biological environment but also attributed to their poor rhizospheric competence, therefore, a careful choice of different conditions is necessary if meaningful data are to be generated (Compant et al., 2005).

About 20 P. fluorescens isolates were isolated from rhizoplane soil of healthy tomato plants and in vitro assays like dual culture, physiological, morphological biochemical and molecular characterization conducted were used to screen and select potential biocontrol agents and subsequently test their ability to suppress fusarium wilt of tomato under greenhouse and field conditions. These results were in line with the findings of Frommell et al. (1991) who isolated 29 Pseudomonas spp from rhizosphere of tomato and tested for their ability to reduce growth of F. oxysporum f.sp. lycopersici and R. solani in vitro.

Pseudomonas spp. has been reported as being antagonistic to several plant pathogenic organisms such as, Macrophomina phaeosolila (Hussain et al., 1990), Pythium sp. (Ongena et al., 1999), F. oxysporum f.sp. lycopersici (Akkopru and Demir 2005), Rhizoctonia solani (Aziz et al.1997), Sclerotium rolfsii (Elad et al., 1980), Phytophthora capsici (Ahmed et al. 1999), Gaeumannomyces graminis var. tritici (Hamdan et al., 1991).

In our study, while screening P. fluorescens isolates for their antagonistic activity against F. oxysporum, all antagonists showed varying levels of inhibition. The percentage of in vitro mycelial growth inhibition by the rhizobacterial isolates against F. oxysporum varied between 7% and 66.3%. Some P. fluorescens isolates were found to be highly inhibitory of F. oxysporum growth where as others showed only mild activity or no activity at all. This suggests that the mode of action exerted and/ or the type of antifungal metabolite produced by the isolates may vary (Williams and Asher, 1996). For the isolates that caused prominent inhibition of fungal
growth in the dual culture experiment, the inhibition zone formed was of such size that there was no physical contact with the pathogens. Suggesting that the rhizobacteria could be producing certain antifungal metabolites (AFMs) (Montealegre et al. 2003). Moreover, as the PDA medium used for the dual culture assay is rich in nutrients, competition might be excluded as the mode of action for these isolates (Landa et al., 1997). The antifungal metabolites produced seem to vary among the isolates tested in this study. In the dual culture assay for instance, some of the isolates not only inhibited the mycelial growth but also changed the appearance of the mycelia from white to reddish brown or red as was evident for isolates. This suggests that the fungal mycelia might have been inhibited not only by antibiosis but also by other antifungal metabolites such as siderophores, hydrogen ions and gaseous products including ethylene hydrogen cyanide and ammonia (Williams and Asher 1996; Kumar et al., 2002; Saravanan et al., 2004). Furthermore, the efficacy of a given biological control agent mostly results, not only from a single mechanism but from a combination of different modes of actions (Alabouvette et al., 1993).

Maximum growth of *P. fluorescens* occurred only at an iron concentration of 50 ppm. Therefore, we confirm that *P. fluorescens* growing in iron-limited conditions (less than 50ppm) should induce siderophore production. *P. fluorescens* bacteria showed growth characteristics, which are compatible to the conditions of salinity, pH, Fe$^{3+}$, and temperature that can be found in soils where tomato plant is commonly cultivated.

Plant growth promoting rhizobacteria (PGPR) have been shown to enhance plant growth and protect roots from invasion by pathogens by a variety of mechanisms including production of antibiotics, hydrogen cyanide, siderophores, and induced systemic resistance (Kleopper et al., 1980; Weller, 1988; Zehnder et al., 2001. Attempts were also made by several researchers to isolate PGPR with specific plant growth promoting traits such as, root colonization (Silva et al., 2003), IAA production (Brie et al., 1991), Phosphate solubilization (Pikovskaya, 1948), Phosphate solubilization and Indole acetic acid production (Hariprasad et al., 2009), Siderophore production (Schyvn & Neilands, 1987), rhizobacteria showing ACC deaminase activity (Pentose and Glick, 2008), Chitinase (Renwik et al., 1991) etc. In our study these traits were found positive under *in vitro* conditions. An effective biocontrol depends on the efficiency of root colonization (Chen et al., 2000).
Currently our knowledge of *P. fluorescens* isolates indicates that it produces high levels of siderophores, which can play an important role in inhibition of *F. oxysporum*. Although bacterial siderophores differ in their ability to sequester iron, they inhibit growth of fungus since the siderophores from pathogen have lower affinity (Loper et al., 1999; O’Sullivan and O’Gara 1992) and the bacterial siderophore-iron complexes can be exploited by plants (Sharma et al., 2003). Siderophore production under iron stress conditions also confers an added advantage for PGPR, in inhibiting pathogen growth or metabolic activity (Henry et al., 2008).

In most biocontrol investigations, a large number of antagonists are commonly isolated in a short period of time and screened *in vitro*. Mycelial inhibition and root colonization do not always correlate with biocontrol efficacy under natural conditions (Paulitz et al., 1992; Williams and Asher, 1996). All promising isolates from the current study were therefore further evaluated under greenhouse and field experiments (objective 4 and chapter 4).