1. INTRODUCTION

Plant rhizosphere is rich in microbial diversity and harbours a variety of microflora, comprising of both beneficial and deleterious components that have the potential to significantly influence plant growth. The beneficial microbes include symbiotic rhizobia, actinomycetes, mycorrhizal fungi and free-living bacteria. Plant growth promoting rhizobacteria (PGPR) and endophytes are a group of rhizospheric bacteria that competitively colonize plant roots and stimulate plant growth and yield and/or reduce plant disease incidence (Dutta and Podile, 2010; Bhattacharyya and Jha, 2012; Khan et al., 2016). PGPR is a wide group which includes various genera such as *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Paenibacillus*, and *Pseudomonas* spp. (Saharan and Nehra, 2011; Goswami et al., 2016). The mechanisms of action involves the phytohormone release (indole-like compounds, cytokinins), antibiotics (phenazine compounds), volatile phyto-stimulants (ethylene and 2,3-butanediol), mineral solubilisation (phosphorous and nitrogen) and ISR through signalling (Haas and Défago, 2005; Saharan and Nehra, 2011; Khan et al., 2016). Due to the aforementioned characters, PGPR are the most popular microbial agents for plant disease management. The bacterial genus *Pseudomonas* is a significant diverse group of PGPR which is specifically take part in plant disease suppression and exhibits plant growth promoting traits (Saharan and Nehra, 2011). The soil-borne fluorescent pseudomonads receives particular attention due to its catabolic versatility, exceptional root colonizing abilities and production of extensive range of antifungal metabolites (Saraf et al., 2014; Parray et al., 2016) and is the extremely diverse and ecologically significant crowd of bacteria on earth (Spiers et al., 2000).

The fluorescent pseudomonads are categorized in within the *Pseudomonas sensu stricto* (rRNA group I), which includes all species *Pseudomonas* which can produce fluorescent siderophores (mainly hydroxamate type). The major representatives of the group includes *P. aeruginosa*, *P. putida*, *P. syringae* and *P. fluorescens* (Couillerot et al., 2009). *Pseudomonas* spp. which can form fluorescent colonies are well known plant disease protection agents and a major contributor to sustainable agriculture and are collectively known as ‘fluorescent pseudomonads’ or ‘fluorescent *Pseudomonas*’ (Babu et al., 2015). The production of siderophores, auxin
compounds, antibiotics, quorum sensing signals, mineral solubilisation etc are the major growth promoting and disease control traits exhibited by the fluorescent pseudomonads (Saharan and Nehra, 2011). These secondary metabolites enable fluorescent pseudomonads to contribute to specific resistance in the host, which can lead to inhibition of phyto-pathogens.

_Fusarium oxysporum_ causes vascular wilt on a wide spectrum of plant species all around world, especially tomato in subtropical climatic conditions (McGovern, 2015). The occurrence of fusarium wilt is the most difficult condition in soil, especially in the deficiency of efficient, chemical, physical and biological management strategies (Bennett _et al_., 2011). Several agronomic strategies have been recommended to manage _Fusarium_ however most of them have failed due to its limitation and side effects. Indeed, induction of host resistance using PGPR is one of the best approaches for the infection management in plants (McGovern, 2015).

Under natural conditions, most of the PGPR mechanisms exerted for growth enhancement of plants are common, whereas under stress conditions, some strains may not be capable to perform efficiently because they fail to compete and survive in adverse environments (Parray _et al_., 2016). Therefore identifying such strains are important in implementing an efficient biocontrol mechanism. Hence, the present objective has been aimed towards isolating, characterizing and shortlisting potential fluorescent pseudomonads, analyse their complex interaction in the light of plant growth promoting features, their antagonistic behaviour against _F. oxysporum_ and siderophores characterisation according to the research model proposed by Latour _et al_. (2003).
2. MATERIALS AND METHODS

2.1. Isolation and Characterization of Fluorescent Pseudomonads

2.1.1. Site Description, Soil Sampling and Isolation

Rhizosphere samples of eight disease-free plants from different locations of India (Mysore, Idukki, Aurangabad, Gangtok and Bangalore) were collected randomly. Soil samples adjacent to the root regions of healthy plants was collected in sterile plastic bags and transferred to the laboratory. One gram of fresh soil sample was added to 9 mL of 0.9 % (w/v) NaCl and serially diluted (10\(^{-1}\) to 10\(^{-6}\)). King’s B (KB) medium was prepared with increasing concentrations of the 8-hydroxy quinoline (0, 40, 60, 80, 100, 120 and 200 mg L\(^{-1}\)) obtained from HiMedia, India (Fallahzadeh-Mamaghani \textit{et al.}, 2009). 8-hydroxyquinoline was heat dissolved in 70 % (v/v) ethanol and added to the KB medium, autoclaved and poured into petri plates under sterile conditions. Addition of 70 % ethanol without 8-hydroxy quinoline served as control (hereafter mentioned 0 mg L\(^{-1}\)). 10 µL of the clear supernatant from serially diluted rhizosphere soil samples were inoculated on to KB plates by spread plating method. Sealed plates were incubated at 37 ± 2 °C for 36 h, observed for fluorescent colonies of pseudomonads under UV light (\(\lambda_{\text{max}} = 365\) nm). Fluorescent colonies were counted, documented and further sub-cultured on KB medium to ensure the purity and were preserved at 4 °C for further characterization.

2.1.2. Morphological and Biochemical Characterisation

All pseudomonad isolates were subjected to their morphological characteristics along with their biochemical characteristics according to Bergy’s manual of systematic bacteriology (Holt \textit{et al.}, 1994). The isolates were also observed for their proficiency to produce oxidase, amylase, gelatinase, urease, caseinase, catalase, hydrogen sulphide and organic acids (Cappuccino and Sherman, 1992).

2.1.2.1. Bacterial morphology and Gram’s nature: Gram’s staining was carried out with heat-fixed bacterial smears prepared from isolated single colonies (Holt \textit{et al.}, 1994). The morphological pattern of the colonies and gram’s nature of the fluorescent pseudomonads were observed under 40X and 100X magnifications using light microscope.
2.1.2.2. **Oxidase test:** Fluorescent pseudomonads were cultured on nutrient agar (HiMedia) plates and incubated for 48 h. Few drops of freshly prepared 1 % (w/v) N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (HiMedia) was added onto the culture plates and observed for the blue colour formation within 10 sec.

2.1.2.3. **Test for production of organic acids:** The fluorescent pseudomonads were inoculated into the fermentation medium prepared with 0.5 % (w/v) glucose (Fisher Scientific). The tubes were then observed after 48 h for colour change of phenol red indicator from red to yellow.

2.1.2.4. **Amylase test:** Fluorescent pseudomonads were streaked on to starch agar plates and incubated for 48 h at 37 ± 2 °C. Lugol’s iodine solution was poured onto plates and observed for a clear zone around the bacterial growth line.

2.1.2.5. **Gelatinase test:** Fluorescent pseudomonads were stab inoculated into tubes containing nutrient gelatin medium and incubated at 37 ± 2 °C for seven days along with uninoculated control tubes. After incubation, the test tubes were placed at 4 °C for 15 min and observed for liquefaction.

2.1.2.6. **Urease test:** Fluorescent pseudomonads were inoculated into Stuart’s urea broth containing yeast extract (0.1g L⁻¹), KH₂PO₄ (9.1 g L⁻¹), K₂HPO₄ (9.5 g L⁻¹), Urea (20 g L⁻¹) and phenol red indicator. The test tubes were then incubated for 48 h at 37 ± 2 °C, observed for the change in colour to deep pink indicates the production of urease.

2.1.2.7. **Caseinase test:** Plates with agar medium containing casein (5 gL⁻¹) as the sole source of carbon were streaked with the fluorescent pseudomonads under aseptic conditions and incubated for 48 h at 37 ± 2 °C and observed for clear zone of hydrolysis around the bacterial colonies.

2.1.2.8. **Hydrogen sulphide production:** The slants of sulphide-indole medium was prepared, autoclaved and streaked with fluorescent pseudomonads and incubated for 48 h at 37 ± 2 °C. Test tubes were checked for black colouration along the line of inoculation.

2.1.2.9. **Catalase test:** A loopful of overnight fluorescent pseudomonad cultures were transferred to sterile glass slides to which 3-4 drops of hydrogen peroxide (Fisher Scientific) was added and observed for effervescence.
2.2. Analysis of Fluorescent Pseudomonads for *in vitro* Direct and Indirect Plant Growth Promotion Traits

Two *Pseudomonas fluorescens* reference strains were used in the study which are referred as PC1 (*P. fluorescens* PFDWD procured from National Bureau of Agriculturally Important Insects, Bangalore) and PC2 (*P. fluorescens* procured from RomVijay Biotech, Puducherry). PC1 has been reportedly able to promote growth in plants under stress environments (Ashwitha *et al*., 2013) and the PC2 is a commercial biocontrol agent used to manage foliar diseases. Bacterial cell concentration of ~ 1 × 10^8 CFU mL^-1 was maintained throughout the studies.

2.2.1. *Ammonia synthesis:* Ammonia syntheses by fluorescent pseudomonads were tested according to Cappuccino and Sherman (1992). The 24 h old pseudomonad cultures were inoculated in 1 % (w/v) peptone (HiMedia) water and incubated at 37 ± 2 °C for 48 - 72 h. 0.5 mL of Nessler’s reagent (HiMedia) was added and observed for brown yellow colour.

2.2.2. *Hydrogen cyanide synthesis:* All the fluorescent pseudomonads were screened for their ability to synthesise hydrogen cyanide (Lorck, 1948). Isolates were inoculated on to sterile nutrient agar plates amended with glycine (4.4 g L^-1). This was then over layered by a filter paper soaked in 2 % (w/v) NaCO₃ was dissolved in 0.5 % (w/v) picric acid (Salkowsky’s reagent) and incubated for 24-72 h at 37 ± 2 °C (Samuel and Muthukkaruppan, 2011).

2.2.3. *Motility testing:* The fluorescent pseudomonads were stab inoculated into the tubes containing indole-ornithine medium consisting of 0.4 % (w/v) agar as per Murinda *et al.* (2002). After incubation the test tubes were checked for diffused growth, indicating motile bacteria and was visually encoded by scores (+, ++ and ++++) for motility.

2.2.4. *Phosphate solubilisation:* The ability of fluorescent pseudomonads to solubilise the inorganic salt phosphate was qualitatively tested. The isolates were streaked on Pikovskaya’s agar medium and incubated for 5 days at 36 ± 2 °C. Phosphate solubilising ability of the fluorescent pseudomonads was determined from the development of clear zone around the bacterial colony (Wahyudi *et al*., 2011).

2.2.5. *Production of indole-like compounds:* Production of major auxins (indole acetic acid and indole butyric acid) in pseudomonads was analysed quantitatively as
per Patten and Glick (1996). Fluorescent pseudomonads were inoculated in nutrient broth containing 500 µg mL⁻¹ (w/v) L-tryptophan (HiMedia) and incubated for 48 h (28 ± 2 °C). Bacterial cells were sedimented by centrifugation (4,000 rpm, 20 min at 4 °C). The upper layer was mixed with Salkowski’s reagent (in 1:4 ratio) and incubated at 65 ± 2 °C for 20 min. A pink colour development indicated indole production. Absorbance of the mixture was measured at 545 nm using a microplate reader (LISA Plus) against standard indole acetic acid (Pedraza et al., 2004).

2.2.6. Siderophore assay: Quantitative estimation of siderophore have been performed using CAS (chrome azurol S)-shuttle assay method of Ali and Vidhale (2011). The isolates were allowed to grow in succinic acid medium containing KH₂PO₄ (3 g L⁻¹), K₂HPO₄ (6 g L⁻¹), MgSO₄ (0.2 g L⁻¹), (NH₄)₂SO₄ (1 g L⁻¹) and succinic acid (4.0 g L⁻¹) at pH 7.0 and incubated for 36 h at 37 ± 2 °C in shaker at 120 rpm. Tubes containing fermented succinic acid medium was centrifuged at 10,000 rpm at 4 ºC for 10 min. This was followed by mixing of cell free supernatant and 0.5 mL CAS shuttle solution (stock added with 4 mM sulphosalicylic acid to make the working solution) in the ratio 1: 1 and incubated for 60 min. Color change was determined using spectrophotometric analysis (ELICO, India) at 630 nm along with a reference containing uninoculated succinate medium and CAS solution (1:1 ratio). Quantitative siderophore production was calculated using the molar extinction coefficient (0.1 M⁻¹ cm⁻¹) as per Schwyn and Neilands (1987).

2.2.6.1. Biochemical characterisation of siderophores: Acid washed glassware was used for the entire siderophore assay. In order to characterize siderophores 72 h old cultures of isolates were subjected to centrifugation (8000 rpm for 10 min at 4 ºC) and the supernatant was filtered through a 0.2 µ millipore filter (Minisart™, Merck). Filtrate was extracted using equivalent volume of chloroform (Merck) in a separating funnel. The upper layer was separated, evaporated and dissolved in 2 mL distilled water and fractions were separated using thin layer chromatography (TLC Silica gel 60F₂₅₄, Merck, Germany). Band was eluted, dissolved and a spectral scan (using Schimadzu UV-1800 spectrophotometer) was executed. In order to remove the iron which is bound to the siderophores, chloroform extraction was carried out by adding 2 % (w/v) 8-hydroxyquinoline and the upper layer was subjected to UV-visible spectrum analysis (Bhattacharya, 2010).
Hydroxamates were characterized by FeCl₃ assay as per Neilands (1981). To 1 mL of cell free culture supernatant 2.5 mL of 2 % (w/v) FeCl₃ (Fisher Scientific) was added. Formation of red or purple color suggested the presence of siderophores. This was further confirmed with the development of red colour using tetrazolium test (Snow 1954), by addition of a pinch of tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and 2N NaOH to the culture supernatant.

Catacholate siderophores were analysed using FeCl₃ assay of Neilands (1981). 1 mL of cell free culture filtrate was added to 1 mL of 2 % (w/v) FeCl₃ and subjected to UV-visible spectrum analysis.

Carboxylates were characterized as per Shenker et al. (1992). 1 mL of test culture was added to 1 mL copper sulphate (250 µM) and 2 mL of sodium-acetate buffer (pH 4). A UV spectral scan of the reaction mixture was executed at 190-280 nm, wherein specific peak absorption indicates carboxylates.

### 2.2.7. Biofilm formation:

All pseudomonad isolates were examined for their capability to form surface-associated-biofilm as per Yamaga et al. (2010) with minor modifications. 20 μL of overnight bacterial culture in Luria Bertoni (LB) broth (HiMedia) was diluted to ~ 1 × 10⁸ CFU mL⁻¹ (OD = 0.3 at 595 nm), was inoculated into 1.8 mL LB medium in microcentrifuge tubes. The inoculated tubes were closed and incubated in vertical position for 24 h at 37 °C. The bacterial cell pellets were carefully washed twice using distilled water, without disturbing the biofilm and stained with 1.8 mL of 0.15 % (w/v) crystal violet (Fisher Scientific) for 20 min. Excess stain was removed, washed twice and bacterial cell pellets were suspended in 1.8 mL of 95 % (v/v) ethanol. Intensity of colour development was read and results were represented as absorbance at 590 nm.

### 2.3. In vitro Mycelial Inhibition Assay:

The antagonistic ability of the isolated fluorescent pseudomonads against F. oxysporum MTCC-1755 was performed using in vitro mycelial inhibition assay (Zongzheng et al., 2009) using a spore suspension of ~ 2 × 10⁶ CFU mL⁻¹ in 0.85 % (w/v) sterile saline. 10 mL of water-agar medium was poured into plates and allowed to solidify. 10 mL of potato dextrose agar (HiMedia) medium was autoclaved, cooled to 40 °C and to which fungal spore suspension was mixed, further cooled and poured onto basal water-agar layer. As the agar solidified, a well was punched in the centre of the plate and 200 μL of fresh fluorescent...
pseudomonad culture suspension (~ $1 \times 10^8$ CFU mL$^{-1}$) was added. Sealed plates were incubated at 28 ± 2 °C. After seven days, the diameter of fungal inhibition was measured and recorded.

2.4. **Growth Promotion Studies in Tomato by Seed Priming:** Growth promotion ability of fluorescent pseudomonads was checked on tomato seeds (PKM-1, LOT-208) as per ISTA (1993). Pseudomonad isolates were subcultured in KB broth and incubated at 37 ± 2 °C for 48 h and centrifuged at 6,000 rpm for 15 min at 4 °C. Bacterial pellets were resuspended in 0.01 M phosphate buffer (pH 7.0). Concentration of bacterial cells in the suspension was adjusted to ~ $1 \times 10^8$ CFU mL$^{-1}$ (OD=0.3). Tomato seeds were surface sterilized using 4 % (v/v) sodium hypochlorite for 30 sec, followed rinsing in distilled water and air drying. Seeds were primed with 10 mL bacterial suspensions for 2 h, blot dried on blotter discs and plated on blotter sheets saturated with distilled water and incubated in growth chamber for 15 days. Root and shoot length were measured and germination percentage calculated as per Baki and Anderson (1973).

2.5. **Statistical Analyses**

All analyses were carried out in quantitative analyses data have been represented as mean ± standard error (where number of replication, n=3). For shortlisting of the best functional fluorescent pseudomonads, plant growth promotion parameters (direct and indirect) and mycelial inhibition results were subjected to a non-parametric Kendall’s concordance coefficient analysis (Garcia-Gutiérrez et al., 2012) using SPSS 18. Over the nine parametric tests performed for plant growth promotion and in vitro antagonism based on the gathering of the positive results, were assigned scores from + to ++++ to generate ranking of pseudomonad isolate which were used for further statistical analysis. Bivariate principal component analysis (PCA) using PAST 3.01 software was used to analyse the functional multiplicity of the shortlisted pseudomonads.
3. RESULTS AND DISCUSSION

Detailed understanding of plant-microbe interactions help in improving plant health and can play a significant role in sustainable agriculture. Therefore in the current study, biochemical characterisation of the plant growth stimulating traits for 144 fluorescent pseudomonads from eight rhizosphere samples were carried out.

3.1. Isolation and Characterization of Fluorescent Pseudomonads

When all the eight rhizosphere soil samples were diluted and plated in KB medium, it was observed that the highest number of fluorescent colonies in the plates was observed in the absence of 8-hydroxyquinoline. Throughout the study, only the bacterial colonies with ideal characteristics of fluorescent pseudomonads were considered for further analyses. It was perceived that fluorescent pseudomonad colonies showed high intensity of fluorescence, at 120 mg L⁻¹ of 8-hydroxyquinoline concentration (Figure 5). Moreover it was evident that the fluorescent colony number decreased gradually with increasing concentration of 8- hydroxyquinoline in the culture plates (Figure 6). The bacterial colonies in the medium containing more than 80 mg L⁻¹ of 8-hydroxyquinoline was shortlisted for further studies. The hypothesis behind selecting fluorescent pseudomonad was to ensure shortlisting of better siderophore producers, which can impart strong biocontrol mechanism on the host plant. Altogether, 144 fluorescent pseudomonads were isolated from eight different rhizosphere samples (Table 1). The highest number of fluorescent pseudomonads isolates was recovered from the rhizosphere of Curcuma longa.

All 144 fluorescent pseudomonads were biochemically characterised for their ability to produce amylase, caseinase, catalase, gelatinase, urease, oxidase and sulfate and glucose reduction. Results of the biochemical characterization were analysed based on Bergy’s manual which indicated that the isolates belonged to genus Pseudomonas. Biochemical behaviour of shortlisted fluorescent pseudomonads isolates showed a vast diversity in biochemical behaviour. All fluorescent pseudomonad candidates were negative for urease, caseinase and glucose fermentation, whereas all were positive for oxidase. Isolates showed varying results for amylase, gelatinase, catalase and hydrogen sulphide production. Biochemical characteristics of the shortlisted eleven fluorescent pseudomonads are depicted in Table 2.
3.2. Analyses for Various Plant Growth Promoting Traits

3.2.1. Direct Traits

All the isolates were further evaluated for the different traits reportedly associated with direct and indirect plant probiotic traits. All the tested fluorescent pseudomonads including controls were positive for ammonia synthesis, which is an important trait of PGPR that may inhibit pathogens, indirectly resulting in plant growth promotion (Raval and Desai, 2012; Parray et al., 2016).

Despite its strong activity as a metabolic inhibitor, hydrogen cyanide synthesised by rhizobacteria does not harmfully affect the host plant, rather it can function as a fungal inhibition agent (Saharan and Nehra, 2011; Parray et al., 2016). Sixty seven fluorescent pseudomonads showed hydrogen cyanide synthesis as showed by the change in color of the filter paper (from orange to brown after incubation). In the current study, isolates with antagonistic activity did not show any correlation with hydrogen cyanide synthesis or siderophore production. Hence, it can be speculated that production of bacterial antibiotics subsequently leads to fungal inhibition. Majority of the fluorescent pseudomonads are excellent secondary metabolite producers, some of which are antifungal agents (Zhuang et al., 2013; Khan et al., 2016; Parray et al., 2016).

A significant amount of insoluble phosphate in soil is in an unavailable form for direct utilization by the plants. Mineral solubilisation is carried out through different mechanisms in PGPR. By the action of PGPR, this unavailable form of minerals is into mono and dibasic form of phosphate through acidification and chelation (Patil et al., 2016). However due to the inconsistent results, the commercial relevance of phosphate-solubilising PGPR has been quite limited (Glick, 2012). Altogether, out of 144 fluorescent pseudomonads used in the current study, 63 were identified as strong phosphate solubilisers. This indicates that the identified phosphate-solubilising fluorescent pseudomonads can potentially help in plant growth through phosphate mineralisation.

The effect of fluorescent pseudomonad treatment on tomato seeds was evaluated on 15th day. Visually it was evident that seed treatment has augmented the root length and germination percentage, which showed that seed priming resulted in a significantly influenced vigour index. Seedlings from seeds primed with the isolate
M140 showed highest root length of 20.96 ± 1.55 cm, germination of 100 % and vigour index of 1363. This was followed by the tomato seed treatment with isolate R68 (root length 20.24 ± 0.98 cm, 100 % germination and vigour index of 1266). The seeds primed with M138 showed an average root length of 20.23 ± 1.53 cm, 100 % germination and vigour index of 1310. Seeds primed with PC1 showed root length of 14.94 ± 0.96 cm, 100 % germination and vigour index 1197.4, whereas seeds primed with PC2 showed root length 17.2 ± 0.94 cm, 100 % germination and vigour index 1051.4) against untreated control (root length 9.73 ± 2.41 cm, 84 % germination and vigour index of 529.2). This indicated that fluorescent pseudomonads have a positive impact on the test plant (Figure 7). Studies have shown that the rhizosphere associated beneficial bacteria, documented as PGPR, foster plant growth through multifaceted mechanisms (Saharan and Nehra, 2011). Direct effects encompass enhanced nutrient availability, stimulated root expansion and/or ISR. The indirect advantageous effects of PGPR entail competition or antagonism towards phytopathogens (Bruto et al., 2014).

In the current study, analyses of in vitro production of indolic compounds showed that 63 out of 144 pseudomonads were positive. Quantitative analyses showed maximum production of indole-like compounds was by the isolate M137 (17 ± 0.96 µg L⁻¹), C2b1 (15.4 ± 2.15 µg L⁻¹) and P96 (15.1 ± 0.90 µg L⁻¹) whereas PC1 and PC2 showed a production of 9.1 ± 0.89 and 6.4 ± 0.75 µg L⁻¹ respectively. Production of indole-like compounds is one of the mechanisms through which the PGPR augments host growth and development. Bacterially synthesised auxins such as indole acetic acid can increase root surface area and length, thereby providing plant greater access to soil nutrients (Glick, 2012). The percentage positivity of various direct plant growth promotion traits are depicted in Figure 8.

Most PGPR isolates synthesise indole-like compounds and which can act as an external growth promoting agent in the host plants (Patten and Glick, 2002). Among the types of phytohormones secreted by, auxins are the major one and quantitatively the most abundant. Indole acetic acid generally exert a positive impact on the host plant through augmenting cell division, extension and differentiation (Lee and Lee, 2010). In addition, these auxins reportedly play a major role in bacterial quorum sensing (de Bruijn et al., 2013). Rhizobacterial synthesis of compounds such as indole
acetic acid, gibberellins etc results in increased length, surface area, and number of root tips, leading to improved nutrient uptake and resulting in improving plant health (Choudhary et al., 2015).

### 3.2.2. Indirect Traits

Fluorescent siderophores are unique to fluorescent pseudomonads - an inherent trait which implicated them as the most efficient PGPR (Upadhyay and Srivastava, 2010) and many of the siderophore-producing bacteria have been used as biocontrol agents to combat phyto-pathogens (Choudhary et al., 2016; Khan et al., 2016). Qualitative CAS assay of 144 fluorescent pseudomonads showed that 58 isolates were showing consistent positive results for the siderophore production (Figure 9a). The highest siderophore accumulation of $8.14 \pm 0.2$ was shown by the isolate M140, followed by $8.02 \pm 0.03 \mu M$ by M137 and $5.98 \pm 0.1 \mu M$ by M132. PC1 and PC2 showed $5.14 \pm 0.3 \mu M$ and $3.18 \pm 0.02 \mu M$ respectively. A shift in absorption maxima was observed in the range of 400-420 nm on addition of FeCl$_3$ stipulating the presence of hydroxamate type of siderophores produced (Figure 9b). This was further confirmed by the formation of red colour on addition of tetrazolium salt. Further chemical characterization showed absence of absorption peaks at 480 and 190-280 nm range which confirmed the complete absence of catecholates and carboxylates respectively affirming that the siderophores produced, were of hydroxamate structure.

Microbially synthesised siderophores accelerate plant growth either directly (by increasing the iron sequestration in the rhizosphere) or indirectly (by competitive inhibition of plant pathogens through competitive iron sequestering). Synthesis of hydroxamate siderophores by fluorescent pseudomonads are advantageous for its utilisation in agriculture as they are comparatively durable and has high iron chelating ability. Nature of siderophore has been found to be independent of the microbial habitat (Kloepper et al., 1980). Usually, pyoverdins are the most prevalent siderophores produced by Pseudomonas with peak absorption between 365-410 nm range (Bhattacharya, 2010). This confirms that the siderophores synthesised by fluorescent pseudomonads were hydroxamates.

Rhizosphere competence and adaptation is a critical feature for any biocontrol strain. Therefore in this study, quantitative analysis of biofilm formation of
fluorescent pseudomonads was considered as criterion for assessment. Significant biofilm forming ability was observed with the isolate T16 (11.49 ± 0.16) followed by T78 (11.24 ± 0.25) and P84 (11.22 ± 1.28) whereas 8.1 ± 0.80 and 7.64 ± 1.3 was shown by PC1 and PC2. Bacterial biofilms can change the nature of its social interactions; which can lead to aggressive colonization through densely packed bacterial population over a surface.

The microbial colonization along with considerable cooperation and competition between microbial cell populations are formed by biofilms (Nadell et al., 2009). High biofilm-forming ability of pseudomonads depicts the ability of isolate to persist in highly competent host rhizosphere. Some fluorescent pseudomonads produce phenazines (a group of small, redox-active compound with antibiotic nature) that influence biofilm development across a large scale, but in unique ways for different Pseudomonas species (Ramos et al., 2010). Although biofilm formation is considered as a necessary process in signal cross talk, reports reveal that non-signal producers also exist in microbial populations which are benefitted from quorum signal producers and hence can reduce the thickness and quality of biofilms (Popat et al., 2012). Overall, multiple characteristics such as quorum sensing, denitrification, siderophore-mediated iron acquisition, substrate utilization etc have been identified in association with rhizosphere competence proficiency of fluorescent pseudomonad strains (Ghirardi et al., 2012).

3.2.1.1. In vitro Mycelial Inhibition Assay

In the current study, the antagonist activity of isolated fluorescent pseudomonads against F. oxysporum (MTCC1755) was tested by well-diffusion assay, which displayed large variability in fungal antagonistic response (Figure 10). Plates were examined after 7 days post inoculation and the radius of inhibition zone was measured. Among the tested 144 isolates, 42 showed different levels of antagonistic behaviour against Fusarium. Highest detected zone for antifungal activity against Fusarium was shown by the isolate R69 (59 ± 6.24 mm) followed by M80 (55 ± 5.29 mm) and T109 (53 ± 8.54 mm) whereas reference strains PC1 and PC2 showed moderate antifungal activity (34 ± 4.36 and 31 ± 6.08 mm respectively). The percentage positivity of various indirect plant growth promotion traits of isolates are depicted in Figure 11.
Ability to inhibit another organism by production of various secondary metabolites is a major necessary trait for potential biocontrol strains. Previous studies have clearly showed that *in vitro* fungal inhibition assay can be used as an effective technique while screening for potential biocontrol agents. Study by Patil *et al.* (2016) used *in vitro* fungal inhibition assay against *Magnaporthe grisea* for the shortlisting of fluorescent pseudomonads. Study found only 61% fluorescent pseudomonads exhibited antagonistic nature against *M. grisea*. Another study by El-Sayed *et al.* (2014) used 531 PGPR isolates against *F. oxysporum* and *Sclerotinia sclerotium*. It was found that 66 strains exhibited efficient inhibition of the fungal pathogens. These studies are in agreement with present observations that the antagonistic nature is not a common trait among PGPR strains. In another noted study by Agaras *et al.* (2015) reported that the antifungal activity of fluorescent pseudomonads are highly versatile in nature. The study identified seven fluorescent pseudomonads which inhibited 12 major fungal pathogens under *in vitro* conditions.

### 3.3. Functional Shortlisting of Fluorescent Pseudomonads using Non-parametric Statistical Assay

A collection of 144 fluorescent pseudomonad strains from rhizospheric soil samples, collected from eight different sites were shortlisted based on plant growth promoting traits. The isolates in the current study were ranked based on non-parametric Kendall’s concordance coefficient test to shortlist the potentially outperforming fluorescent pseudomonads, where rank 1 is considered highest. Eleven best isolates with rank ranging from 1-10 have been shortlisted for further studies. PC1 and PC2 were ranked 9 and 3 respectively whereas M140 was the best with rank 1, followed by M139 (Table 3). The shortlisted fluorescent pseudomonads were R69 (isolated from rice rhizosphere), P78 (isolated from paarijatham rhizosphere), T109 (isolated from tomato rhizosphere) and M80, M96, M127, M132, M135, M137, M139 and M140 (isolated from maize rhizosphere).

Bivariate PCA of the shortlisted isolates by the cross validation method showed that all eleven fluorescent pseudomonads were distributed towards right side (Figure 12). Component 1 and 2 showed 60.61 and 16.14 percentage variance respectively with a highest Eign value of 12.10, which is a positive sign for the vast functional multiplicity of the fluorescent pseudomonads. However, the overall trend
of PCA graph towards the right side was in agreement with biofilm, indole and siderophore production.

Use of PGPR to foster soil fertility and enhance growth and yield of agronomically important crops is a consequential substitute to chemical fertilizers in sustainable agriculture (Stajkovic et al., 2011). Study on Arabidopsis clearly showed that presence of bacterially synthesised indolic compound does not necessarily change \textit{in vivo} plant hormone levels (Schwachtje et al., 2012). Some of the isolates exhibited root augmentation traits in tomato seedlings even without production of indole-like compounds, which may be likely due to the significant biofilm formation and siderophore production. It was observed that all the fluorescent pseudomonads forming biofilm, showed root growth promotion in tomato seedlings. With respect to the considered plant growth promotion traits, primed seeds showed enhanced germination with respect to distilled water treated control seedlings. Root growth promotion is one of the major-established beneficial effects for PGPR. Rapid establishment of roots is advantageous for young seedlings, enhancing their probability of survival, as it improves their ability to anchor in the soil to gather water and nutrients from surroundings.

In this study, the isolates with observed abundance of indole-like compounds did not result in significant root growth promotion in tomato seedlings. However, fluorescent pseudomonads with moderate indole-like compound production resulted in considerable growth enhancement in tomato. Endogenous indole-like compounds of host may be optimal or suboptimal in roots. Bacterially synthesised indole-like compounds may alter indole level in host plant, hence resulting in plant growth promotion or even inhibition (Glick, 2012). In this regard, level of indole-like compounds synthesised by the plant is important in determining whether bacterial indole-like compounds stimulate or suppress plant growth. It was shown that the amount of indole acetic acid produced by a bacterium is dependent on its respective gene location and type of pathways. Precursor gene for indole acetic acid synthesis is located in plasmids, can result in high production unlike in chromosome location, as the replication and numbers of plasmids are independent of the core bacterial genome (Duca et al., 2014).
All the tested fluorescent pseudomonads displayed moderate to high motility under laboratory conditions. De Weert et al. (2002) established that motility in fluorescent *Pseudomonas* is a major attribute towards root colonization and organic acids such as malic acid and citric acid are among major chemo-attractants for *P. fluorescens* in tomato rhizosphere. This study has been backed by Choi et al. (2006) that biofilm development and swarming motility may contribute to bacterial colonization and stable maintenance of antifungal compounds on plant surface.

Secondary metabolites such as benzoxazinoids liberated from the plant roots can attract many soil bacteria playing a major role in chemotaxis (Neal et al., 2012). It has been reported that PGPR can produce multi-mechanism system of defence through production of various, lytic enzymes, volatile and diffusible defence compounds that may play a major role in the observed fungal inhibition (Selvakumar et al., 2013; Choudhary et al., 2016; Khan et al., 2016).

A detailed interpretation of the fundamental mechanisms of these beneficial microbes, which enable their interaction with plant physiology, was critical to achieve efficient biocontrol applications (Choudhary et al., 2016). Therefore in the current study, 144 fluorescent pseudomonads were tested for their characteristic traits, such as motility, phosphate solubilisation, synthesis of ammonia and hydrogen cyanide, production of phytohormones and siderophores resulting in root augmentation and the antagonistic nature against *Fusarium*. Previous studies have shown that PGPR exerts specific growth promoting effects on plants, especially on hosts, presumably developed during long-term co-evolution. Nevertheless, among the fluorescent pseudomonads subjected to investigation, eighteen that were from tomato rhizosphere, did not show significant growth promotion in tomato plants, except T109 which was ranked 10. All the other shortlisted pseudomonads were isolated from different plant rhizospheres. In addition, shortlisted pseudomonads did not show any host dependent growth promotion. Although various studies have put forth different theories that affect root colonization of PGPR, major factors include species, developmental stages and soil types. These have been indicated as key elements determining the configuration of rhizosphere-microbial communities. Rhizosphere of *Nyctanthes arbor-tristis* showed significant number of isolates which possess appreciable plant growth promotion traits. It is also worthy to point out that the
conventional hunting for rhizosphere bacteria were restricted to the agriculturally important short living plant species. However, the present results depicts that other plants also harbours rich diversity in rhizosphere microorganisms with potential isolates and deserves more scientific attention.

In the current objective, efficient isolation techniques along with various plant growth promoting traits of fluorescent pseudomonads have been considered. The results indicate that the antagonistic activity and the role of these isolates in induction of resistance need to be explored in green-house conditions. The study demonstrates the capability of fluorescent pseudomonads to stimulate growth in tomato plants and antagonize *F. oxysporum* MTCC1755. It must be emphasised that the proficiency of fluorescent pseudomonads to subvert fungal pathogens is a highly encouraging way to modulate the plant defence strategies, in terms of competitive survival in rhizobiome. Overall, eleven shortlisted fluorescent pseudomonads were shortlisted based on their plant beneficial traits, and stored for further analyses.