

Biofilm Development, Plant Growth Promoting Traits and Rhizosphere Colonization by *Pseudomonas entomophila* FAP1: A Promising PGPR

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Abstract

Among the diverse soil bacteria, plant growth promoting rhizobacteria (PGPR) mark an important role in enhancing plant growth through a range of beneficial functions. This is mainly achieved by effective rhizosphere colonization by PGPR. Biofilm development by PGPR is considered as a survival strategy over the planktonic mode of growth under stress and natural conditions. Since the performance of microbial inoculants under field conditions is not always consistent due to various biotic and abiotic factors affecting survival, colonization and functions. Therefore, the rhizobacteria with efficient colonization ability and exhibiting multiple PGP traits are expected to perform better. We hypothesized that the biofilm forming ability of PGPR on plant root will be an added advantage to rhizosphere colonization. Therefore, we have selected a promising isolate of PGPR through random screening programme from rhizoplane of wheat (*Triticum aestivum*). The selection was based on biofilm development ability, multifarious PGP activities (production of indole acetic acid, siderophore, phosphate solubilization, hydrogen cyanide, ammonia production and biocontrol activity) and tolerance to salinity and heavy metals. The selected isolate was identified by 16 s rRNA partial gene sequencing as *Pseudomonas entomophila*-FAP1. The strain FAP1 formed strong biofilm in microtitre plate, glass surface as well as on the roots of wheat seedlings. Biofilm forming capacity of the FAP1 was characterized by scanning electron microscopy and confocal laser scanning microscopy. FAP1 exhibited biofilm-related traits such as the production of exopolysaccharides, EPS ($1501.33 \pm 1.08 \mu\text{g ml}^{-1}$), alginate ($212.81 \pm 1.09 \mu\text{g ml}^{-1}$), swarming motility ($22 \pm 1.36 \text{ mm}$), swimming motility ($31 \pm 2.12 \text{ mm}$) and cell surface hy-

drophobicity (63%). Rhizosphere colonization by FAP1 was found 7.5 Log CFU g⁻¹ of soil comparable with rhizoplane colonization (7.2 Log CFU g⁻¹ of root). Therefore, biofilm formation on plant roots by promising PGPR may be included as an additional criterion to select a better rhizosphere colonizer. Further, study with mutant deficient in biofilm should be developed for comparative study to explore the exact contribution of biofilm in root colonization under natural soil-plant system.

Keywords

Biofilm, PGPR, Rhizosphere Colonization, *Pseudomonas entomophila*, SEM, and CLSM

1. Introduction

Biofilm development by rhizobacteria is considered as a common mode of life-style under the natural condition and provided protection to a number of biotic and abiotic stress conditions. The interaction among various plant growth promoting rhizobacteria has been explored mainly under the planktonic mode of growth. However, understanding on the behavior of PGPR in biofilm mode on the root surface and soil is still in the stage of infancy. Majority of the rhizobacteria are capable of forming biofilm *in vitro* but root-associated biofilm *in situ* has yet to be explored as the conditions are different in the soil-plant root system. Therefore an in-depth investigation of the biofilm development on root surface and rhizosphere colonization is needed to improve the understanding of plant-microbe interaction. Biofilms are bacterial communities enclosed within self-produced extracellular polymeric substances (EPS). In nature, biofilm constitutes a protected growth modality allowing bacteria to survive in hostile environment. Various traits which are associated directly or indirectly to the biofilm development includes EPS production, swarming and swimming motility, cell surface hydrophobicity and alginate production in *Pseudomonas* sp. and other bacteria [1].

The biofilm may influence resource competition or production of inhibitory compounds and increase resistance to abiotic stresses [2]. Beneficial plant growth promoting rhizobacteria (PGPR) associated with plant root surfaces are known to contribute towards increase in plant yield [3]. The mechanisms of plant growth promotion by various PGPR has been well documented by both direct (production of plant hormones, nitrogen fixation, phosphate solubilization and sequestering iron) and indirect (antibiotics and lytic enzymes, induced resistance, HCN production and competition) mechanisms [4] [5] [6]. However, environmental factors influencing the survival and rhizosphere colonization played a key role in ensuring consistency in the performance of PGPR. The role of biofilm has been documented in maintaining ecological balance and plant growth in the natural soil [7] and, compared with planktonic counterparts, conferred benefits such as increased resistance to antibacterial compounds, en-

hanced protection from desiccation and protozoan predation and elevated rates of horizontal gene transfer [8] [9] [10]. The PGPR are known to adhere or colonize in the rhizosphere and stimulate the growth of plants and suppress plant diseases. [11]. There is evidence indicating that inoculation with biofilm-forming PGPR has better plant growth promotion as compared to non-biofilm former inoculants [12]. In another study, [13] demonstrated that the resistance mechanism provided through biofilm mode of growth protected plant from stress condition. Considering the role of biofilm as a bacterial survival strategy, we hypothesized that a promising PGPR strain exhibiting strong ability to form biofilm might be an effective root/rhizosphere colonizer. In this study a promising new isolate of *Pseudomonas* sp. FPA1 was selected to assess its biofilm development and rhizosphere colonization ability to be exploited as promising PGPR for wheat.

2. Materials and Methods

2.1 Bacterial Strains and Their Characteristics

Rhizoplane of wheat (*Triticum aestivum*) was collected aseptically and kept in sterile polybag before isolating *Pseudomonas* sp. on Kings B agar medium (Hi-media) containing per liter of distilled water: 10 g proteose peptone, 10 ml glycerol, 1.5 g K_2HPO_4 , 1.5 g $MgSO_4$, 20 g agar, pH 7.2. Colonies exhibiting characteristics of *Pseudomonas* sp. were selected and purified. The suspected culture was subjected to cultural and biochemical characteristics before assigning an isolate number for preservation and further identification. Promising *Pseudomonas* isolates were selected for our lab culture collection. One of the promising isolate FAP1 was subjected to molecular identification by 16 s rRNA partial gene sequence analysis. The identified isolate was further used in this study. Nutrient broth/agar (Hi-media) was used to culture test isolates.

2.2. Screening Assay for PGP Attributes

Qualitative estimation of Indole acetic acid production (IAA) was detected by the method of [14]. Method of [15] as adopted by [5] was used for quantitative estimation of IAA production in the presence of $500 \mu\text{g ml}^{-1}$ tryptophan. Ammonia production in peptone water was done by using the method of [16]. Production of hydrogen cyanide among test isolates was screened using the method of [17]. Quantitative estimation of siderophore was estimated adapting the method of [18]. Qualitative estimation of phosphate solubilization was done by observing a halo zone around the bacterial colony growing in Pikovskaya medium [19]. Solubilization of tricalcium phosphate in liquid medium quantitatively was performed as described earlier [5]. Antifungal activity was determined by observing clear zone around bacterial colony growing in Potato dextrose agar medium (Hi-media) [20].

2.3. Tolerance to Salt (NaCl), Heavy Metals and Antibiotics

To determine the salt tolerance of the test isolates, they were cultured in KB

broth (King *et al.* 1994) supplemented with 25 - 800 mM salt (NaCl) concentrations and kept at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 48 h. The growth was observed after 48 h of incubation. The test isolates were tested for tolerance to heavy metals by agar plate dilution method using nutrient agar medium treated separately with increasing concentrations ($0 - 1600 \mu\text{g ml}^{-1}$; at a two-fold dilution interval) each of Cu ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), Cd (CdCl_2), Cr ($\text{K}_2\text{Cr}_2\text{O}_7$), Zn (ZnCl_2) and Pb (PbCl_2) were spot inoculated with $10 \mu\text{l}$ of $10^8 \text{ cells ml}^{-1}$. Plates were incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 48 h and the highest concentration of each metal supporting bacterial growth was defined as maximum tolerance level (MTL). Antibiotic sensitivity was assayed by disc diffusion method using Muller Hinton agar medium. Briefly, $100 \mu\text{l}$ of freshly grown and appropriately diluted culture 10^7 CFU ml^{-1} was spread and antibiotic disc were placed on plates and incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 48 h. sensitivity to antibiotic was measured by measuring the zone of inhibition around disc.

2.4. Quantitative Estimation of Exopolysaccharides (EPS) Production

EPS extraction and quantitation were performed as described by [21]. The pre-inoculum was grown in KB broth (Hi-media) for *Pseudomonas entomophila* FAP1 for overnight at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The volume of $500 \mu\text{l}$ of pre-inoculum was added in fresh 50 ml culture medium and allow to grow at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for five days at 120 rpm in the rotatory shaking incubator. The culture volume of 200 ml was centrifuged at 800 rpm for 20 min at 4°C . The supernatant was filtered through nitrocellulose filter with the pore size of $0.45 \mu\text{m}$ (Millipore filter, Bangalore, India). From the final filtrate, EPSs were precipitated after the addition of three volumes of chilled ethanol and the solution was kept at 4°C for overnight for precipitation of exopolysaccharides. The weight of the precipitated EPS was measured after drying at 80°C for 48 h.

2.5. Alginate Quantification Assay

Alginate extraction in test isolates was performed with 48 h grown culture. After incubation, the culture was centrifuged at 10,000 rpm for 10 min and cell-free supernatant was collected. Isolation of deacetylated alginate from culture supernatants was performed by adding an equal volume of isopropanol and kept it for one day under static conditions. The precipitate was collected by centrifugation 10,000 rpm for 10 min. The resultant pellet was washed successively with 1 ml of 70% and 96% ethanol. The pellet was dried at 37°C for 15 min and dissolved in 1 ml of sterile ddH_2O . For quantification, $100 \mu\text{l}$ of the suspension was transferred to fresh test tubes and volume was made up to 1 ml with Milli-Q water. One ml of freshly prepared borate sulfuric acid solution was added followed by $30 \mu\text{l}$ of fresh carbazole reagent and mixed thoroughly. The mixture was kept for 15 min at room temperature and the absorbance was measured at 500 nm against a reagent blank and calculated in terms of $\mu\text{g/mg}$ wet biomass [22]. Every assay was repeated in triplicate at least three times.

2.6. Cell Surface Hydrophobicity Assay

Cell surface hydrophobicity (CSH) was quantified by the using microbial adhesion assay to hydrocarbons (MATH) as described by [23]. Bacterial cultures were incubated in NB broth and hydrophobicity was determined after 1st, 2nd and 4th day of incubation. To evaluate the percentage of hydrophobicity, 5 ml of 24 h grown culture was centrifuged at 8000 rpm for 10 min and pellets were resuspended in phosphate-magnesium buffer (pH = 7.4), and read the absorbance at 400 nm using UV-vis spectrophotometer [Shimadzu UV-vis spectrophotometer-8500 II] called initial bacterial suspension. Five ml of culture was added to 0.2 ml of hexadecane and mixed vigorously with the help of cyclomixer. After separating the aqueous phase, absorbance was determined at 400 nm called as final concentration in the aqueous phase. The percentage of hydrophobicity was calculated as follows:

$$\text{Percent hydrophobicity}(\%) = [1 - (A_1/A_0)] * 100$$

where A_1 is initial bacterial suspension absorbance and A_0 is absorbance of the aqueous phase.

2.7. Swimming and Swarming Motility Assay

Swimming and swarming motility assay was performed according to the method adopted by [24]. Adler (1966). Assay plates containing Nutrient broth medium with 0.3% and 0.5% (w/v) agar were used for swimming and swarming motility assay respectively. Each assay plate was spot inoculated with 3 μ l of the freshly grown bacterial culture of cell density of (10^7 cells ml^{-1}). The inoculated plates were sealed with the help of parafilm to prevent dehydration and incubated at $28^\circ\text{C} \pm 2^\circ\text{C}$ temperature for 48 h. Swimming and swarming motilities were then determined by measuring the swarm diameter after 24 h and 48 h and expressed the zone in millimeters.

2.8. Biofilm Quantification by Use of Crystal Violet Assay

In vitro biofilm quantification was performed by using the method of [25]. Bacteria were grown overnight on Nutrient agar (NA) plate was resuspended in NB medium and diluted to the final optical density at 600 nm of 0.02, bacterial cultures were transferred to 96 well polystyrene plate. The volume of culture was 160 μ l per well. The cultures were allowed to stand at $28 \pm 2^\circ\text{C}$ for 48 h. The quantification of biofilm formation was assayed by the staining with 0.1% crystal violet. After the growth period wells of the microtitre plate was emptied and wash gently with double distilled water at least three times to remove loosely attached bacterial cells and left at room temperature for 30 min. Samples were stained by the addition of the 200 μ l of the 0.1% crystal violet solution to each well and incubated at 28°C for 20 min. The wells of plates were then washed. The intensity of crystal violet staining was measured after addition of 70% ethanol to each dry well. The samples were incubated for 20 min, after which the

absorbance was measured at 590 nm on ELISA plate reader (Thermo Scientific Multiskan EX, UK). Biofilm quantification were tested in five independent wells.

The data were statistically analyzed by analysis of variance test (SPSS version 17.0 for Windows; SPSS Inc., Chicago, IL, USA).

2.9. Bacterial Seed Treatment on Germination and Vigour Index in Wheat

In order to determine the effect of the isolates on germination and seedling vigour, twenty seeds of wheat inoculated with each isolate were incubated in 9-cm petri dishes on two layers of moistened filter paper. As a control treatment, seeds treated with water instead of bacterial inoculum were also observed. In order to maintain sufficient moisture for germination, 5 ml distilled water was added to the petri dishes every other day and seeds were incubated in climate control growth chamber. The germination percentage was recorded every 24 h for 7 days. Root and shoot length were measured after seventh day. The experiment was planned as a completely randomized design with five replication for each isolates.

Germination rate(%) = number of seeds germinated ÷ total number of seeds × 100

Vigour index = % germination × total plant length

2.10. Biofilm Formation on Glass Surface and Visualization by SEM and CLSM

Freshly grown *Pseudomonas entomophila* FAP1 (2 ml culture and 2 ml nutrient broth) was kept in 12 well tissue culture plate. Sterile glass coverslip (20 mm) was placed in each well and incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 to 48 h under static condition. After the incubation period, glass coverslip was washed three times with 1X phosphate buffer saline (pH 7.2) to remove loosely attached cells from the glass surface. The biofilm on the glass surface was fixed using 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 4 h. The samples were dehydrated through a graded ethanol series 30% - 100% (v/v) and shadowed with gold for viewing under a Scanning Electron Microscope (Model: JOEL, 76510LV, Japan) at University Sophisticated Instrumentation Facility, AMU, Aligarh. For Confocal Laser Scanning Microscopy (CLSM), the biofilm formed on the glass surface as mention above and stained with 0.1% acridine orange for 20 min and examined. The depth or thickness of biofilm on the glass surface was analyzed by Z-stack analysis.

2.11. Biofilm Development on Root Surface

Biofilm development on the root surface of wheat by FAP1 strain, a separate experiment was planned to assess the biofilm. Briefly, after the transplantation of seven days, two to three leaf stage of *Triticum aestivum* seedling which was dipped in overnight grown culture in the gnotobiotic soil system, plants were uprooted and washed to remove loosely attached cells from the root surface. The

root samples were fixed in 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 2 h according to Smyth *et al.* 1990, The root samples were dehydrated through a graded ethanol series (30% - 100%, v/v) and critical point dried in CO₂. To prevent tissue damage pressure was released slowly and shadowed with gold (22 nm) before viewing under Scanning Electron Microscope (Model: JOEL, 76510LV, Japan).

2.12. Rhizoplane and Rhizosphere Colonization

The method described by [26]. was adapted for root colonization with little modifications. Briefly, sandy-clay-loam soil (250 gm) in an “autoclavable plastic pots” was maintained in a climatic-controlled growth chamber. Plant nutrient solution 10% (vol/wt) was added to moisten the soil. For root colonization experiment, seven days old aseptically grown wheat seedling which was two to three leaf stage were dipped into overnight grown culture for four hours and transplanted to sterile soil microcosm in small plastic pots and kept in the climate controlled growth chamber. After each day intervals of 1 - 15 days after transplantation (DAT), seedling uprooted and washed gently in sterile double distilled water to remove loosely attached cells from the root surface. Root (1 gm) removed from seedlings and macerated using mortar pestle aseptically and appropriately diluted in sterile normal saline solution (NSS). The CFU was determined by plating 100 µl of the sample on nutrient agar plates amended with 30 µg/ml of rifampicin for selective isolation and incubated at 28°C ± 2°C for 48 h. (Zhang *et al.* 2011). Furthermore, on the same time intervals, (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 days), the rhizospheric soils were collected which was tightly adhered to the root surface and serially diluted into the normal saline solution (NSS). The volume of 100 µl of each dilution was plated on KB agar medium. Plates were incubated at 28°C ± 2°C for 48 h and CFU/gm of soil was counted.

2.13. Statistical Analysis

All experiments were performed at least three times independently with three to five times replication. Statistical analyses were performed using SPSS (Version 17) for LSD calculation and Microsoft Office Excel 2007 used to graphics. Pot experiments were performed in completely randomized design with 3 - 5 replications.

3. Results

3.1. Characterization of Plant Growth Promoting Activities, Biofilm Screening and Identification of Test Bacteria

The *Pseudomonas* strains were isolated after enrichment on King's B medium exhibiting characteristics cultural, morphological and fluorescens at 254 nm under UV-illuminator was selected with smooth edge and convex shape surface and tested isolates reacted negatively to the Gram's reaction. The biochemical characteristics relevant to *Pseudomonas* sp. has depicted in **Table 1**. All the iso-

lated strains were grown at 4°C but no growth at 41°C. Additionally, *Pseudomonas* sp. showed positive result for catalase and oxidase test. Further selected isolates were screened for their multiple PGP activities, tolerance to salt, heavy metals and antibiotics. Based on promising multifarious PGP activities and tolerance to abiotic stresses and biofilm formation, isolate FAP1 was selected for further study. The FAP1 produces Indole acetic acid, siderophore, HCN, phosphate solubilizing, and antifungal activity was also demonstrated. The FAP1 strain had also shown tolerance to heavy metals, antibiotics and salinity stresses. *In vitro* biofilm development on microtitre plates indicated FAP1 formed a strong biofilm as presented in **Table 1**.

Isolate FAP1 was further identified on 16 S rRNA partial gene sequencing at DNA Sequencing Service (Macrogen Inc., Seoul, South Korea) using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACTT-3'). The resulting nucleotide sequence was submitted to the GenBank Sequence Database (accession number KY110949). The phylogenetic tree was constructed from 16 s rRNA sequence showed 99% similarity with *Pseudomonas entomophila*.

3.2. Quantitative Estimation of PGP Attributes

Quantitative estimation of IAA production in the presence of exogenous supply (500 µg ml⁻¹) of tryptophan revealed that highest in *Pseudomonas entomophila* FAP1 strain (209.25 ± 1.32 µg ml⁻¹). Additionally, other isolates were also showed varying level of the production of IAA as depicted in **Table 1**.

3.3. Alginate and EPS Quantification Assay

Alginate production was found to be highest in the FAP1 strain (212.81 ± 1.09 µg ml⁻¹). However, other isolates also showed varying degree of alginate production as presented in **Table 1**. Production of exopolysaccharides (EPS) was observed to be more in the FAP1 strain (1501.33 ± 1.08 µg ml⁻¹). However, other isolates were also showed varying level of EPS production (**Table 1**).

3.4. Cell Surface Hydrophobicity

In the present study, *Pseudomonas entomophila* FAP1 revealed the adhesion to hydrocarbons and hydrophobicity as: 63%. Although rest of the isolates were also assayed for the hydrophobicity and showed varying pattern in their percent adhesion to hydrocarbon as presented in **Table 1**.

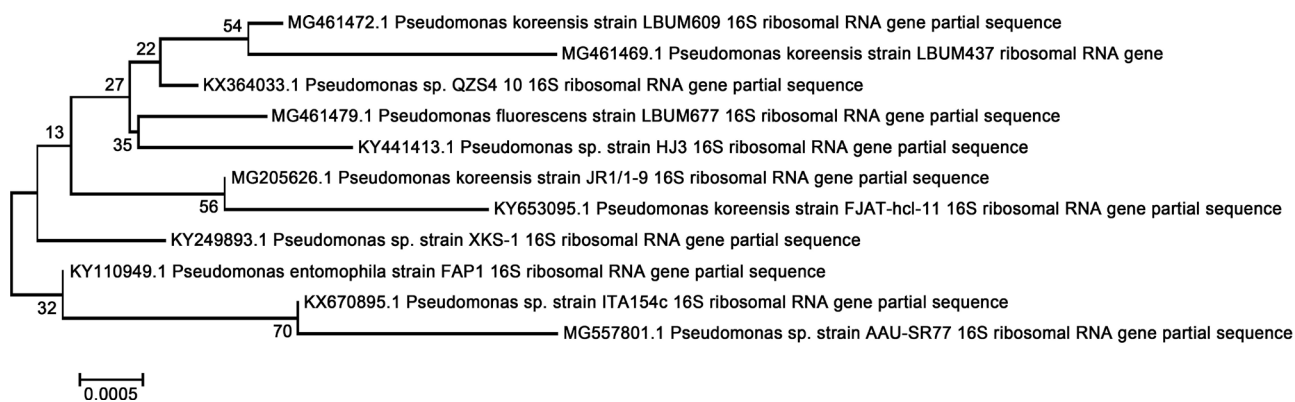
3.5. Swimming and Swarming Activity

After 48 h of the incubation period, we measured the swarm zone of tested isolates. *Pseudomonas entomophila* FAP1 strain showed the swimming motility that was 31 ± 2.12 mm in diameter and swarming motility for FAP1 was observed in the form of swarm zone that was 22 ± 1.36 mm in diameter in the FAP1 strain. However, other isolates were also revealed variation in their swimming

Table 1. Characteristics of biofilm forming stress tolerant plant growth promoting *Pseudomonas* strains under the study.

| PGP traits | PS1 | PS2 | PS3 | PS4 | PS5 | PS6 | PS7 |
|--|---|---|---|---|---|---|--|
| IAA ($\mu\text{g ml}^{-1}$) | 209.25 \pm 1.15 | 149.21 \pm 1.16 | 169.31 \pm 1.26 | 159.21 \pm 1.16 | 189.41 \pm 1.26 | 139.91 \pm 1.66 | 179.81 \pm 1.06 |
| Phosphate solubilization ($\mu\text{g ml}^{-1}$) | +ve (170.45 \pm 1.22) | +ve (120.40 \pm 1.12) | +ve (129.42 \pm 1.22) | +ve (119.32 \pm 1.32) | +ve (123.22 \pm 1.42) | +ve (153.32 \pm 1.52) | +ve (163.42 \pm 1.32) |
| Siderophore ($\mu\text{g ml}^{-1}$) | +ve (23.54 \pm 1.36) | +ve (16.52 \pm 1.16) | +ve (12.42 \pm 1.06) | +ve (17.32 \pm 1.02) | +ve (13.39 \pm 1.32) | +ve (20.49 \pm 1.52) | +ve (10.49 \pm 1.32) |
| Ammonium | + | + | + | - | + | - | + |
| Hydrogen cyanide | + | - | + | - | - | - | - |
| Antifungal activity | + | - | - | + | - | - | - |
| Salt tolerance (NaCl) mM | 800 | 400 | 200 | 200 | 400 | 400 | 800 |
| Heavy metal tolerance | | | | | | | |
| Cd ($\mu\text{g ml}^{-1}$) | 40 | 20 | 40 | 20 | 10 | 10 | 20 |
| Cu ($\mu\text{g ml}^{-1}$) | 400 | 400 | 400 | 200 | 200 | 200 | 400 |
| Cr ($\mu\text{g ml}^{-1}$) | 800 | 400 | 400 | 200 | 200 | 200 | 200 |
| Pb ($\mu\text{g ml}^{-1}$) | 200 | 100 | 50 | 100 | 200 | 100 | 50 |
| Zn ($\mu\text{g ml}^{-1}$) | 800 | 400 | 800 | 200 | 200 | 400 | 400 |
| Antibiotics tolerance* | AZM, AMX, TE, AMP, CTX | LE, TE, NX, NA | AMP, NA | AZM, AMP, DO, NA | AZM, AMX, DO, NA | LE, AMX, NX, NA | AMX, TE, AMP |
| Biofilm and associated traits | | | | | | | |
| Biofilm formation | | | | | | | |
| Swarming motility mm (diameter) | Strong (1.09 \pm 0.25) 22 \pm 1.36 | Moderate (0.69 \pm 0.29) 23 \pm 1.46 | Weak (0.39 \pm 0.19) 19 \pm 1.26 | Weak (0.37 \pm 0.49) 20 \pm 1.29 | Weak (0.31 \pm 0.39) 25 \pm 1.19 | Strong (0.98 \pm 0.29) 17 \pm 1.07 | Moderate(0.68 \pm 0.32) 19 \pm 1.08 |
| Swimming motility mm (diameter) | 31 \pm 2.12 | 33 \pm 2.62 | 29 \pm 2.32 | 24 \pm 2.12 | 29 \pm 2.19 | 22 \pm 2.20 | 24 \pm 1.27 |
| Cell surface hydrophobicity | 63% | 43% | 46% | 36% | 41% | 58% | 39% |
| EPSs production ($\mu\text{g ml}^{-1}$) | 1501.33 \pm 1.08 | 1201.33 \pm 1.09 | 1150.23 \pm 1.03 | 1130.13 \pm 1.09 | 1030.23 \pm 1.19 | 1430.43 \pm 1.30 | 1230.43 \pm 1.50 |
| Alginate production ($\mu\text{g ml}^{-1}$) | 212.81 \pm 1.09 | 189.81 \pm 1.03 | 179.31 \pm 1.08 | 139.32 \pm 1.18 | 119.32 \pm 1.08 | 199.32 \pm 1.38 | 169.32 \pm 1.28 |
| Molecular identification 16 s rRNA partial gene sequencing | <i>Pseudomonas entomophila</i> FAP1 (Figure 1) | | | | | | |

*Antibiotics disc potency used as described, Azithromycin (AZM) 30 μg , Ciprofloxacin (CIP) 30 μg , Levofloxacin (LE) 5 μg , Amoxicillin (AMX) 30 μg , Tetracycline (TE) 30 μg , Ampicillin (AMP) 2 μg , Norfloxacin (NX) 10 μg , Doxycycline (DO) 30 μg , Nalidixic acid (NA) 30 μg and Cefotaxim (CTX) 300 μg .

**Figure 1.** Phylogenetic tree of *Pseudomonas entomophila* FAP1.

and swarming motility as depicted in **Table 1**.

3.6. Microtitre Plate Assay for Biofilm Development

An assay for biofilm formation was performed on 96 well microtitre plates. In this study, crystal violet assay was used to measure the quantitation of biofilm

formation [27]. Rhizobacterial strain *Pseudomonas entomophila* FAP1 showed strong biofilm development on the microtitre plate and optical density measurement was 1.09 ± 0.25 of crystal violet at 590 nm. Although, other isolates were also quantified for biofilm development and showed varying level of biofilm forming ability as represented in Table 1.

3.7. Effect of Bacterial Seed Treatment on Germination and Vigour Index in Wheat

The effect of rhizobacterial treatment upon seed germination and vigour index of wheat varied with different isolates. The FAP1 strain had a significant effect on germination rate and vigour index over all the tested isolates. Seed germination was increased $84.58\% \pm 1.25\%$ and vigour index was $78.25\% \pm 1.36\%$ with the treatment of FAP1 compared to uninoculated control. Although, other isolates were also revealed different pattern of seed germination and vigour index as depicted in Figure 2.

3.8. Biofilm Development on Glass Surface

An assay for biofilm development on the glass surface was performed in 12 well tissue culture plate. The test bacterium FAP1 was formed strong and very dense biofilm on the glass surface as evident from SEM micrograph depicted in Figure 3. Confocal laser scanning micrograph was also provided evidence of strong biofilm development and thickness of biofilm on the glass surface as depicted in Figure 4(a) and Figure 4(b).

3.9. Biofilm Formation on Root Surface

Adherence of bacteria on the root surface of wheat (*Triticum aestivum*) was visualized after incubating seven days old seedling with the freshly grown culture

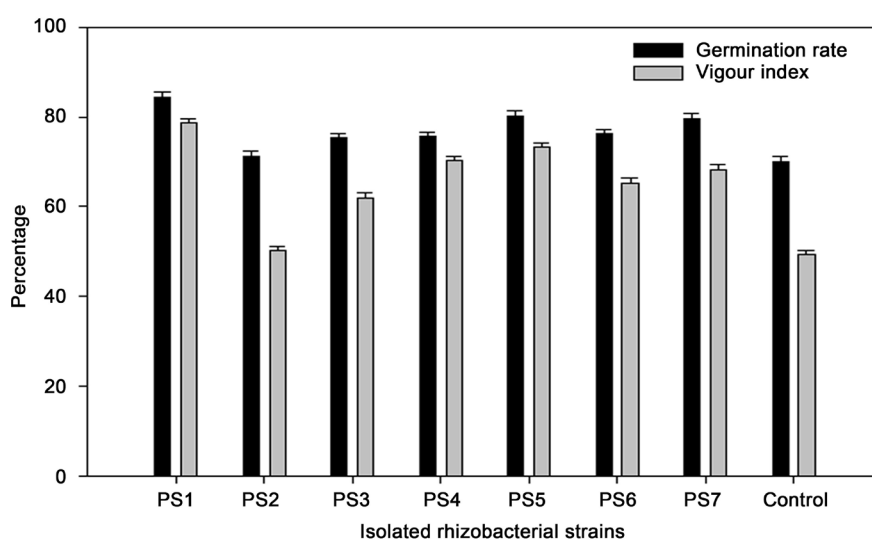


Figure 2. Effect of rhizobacterial treatment on the germination rate and seedling vigour index.

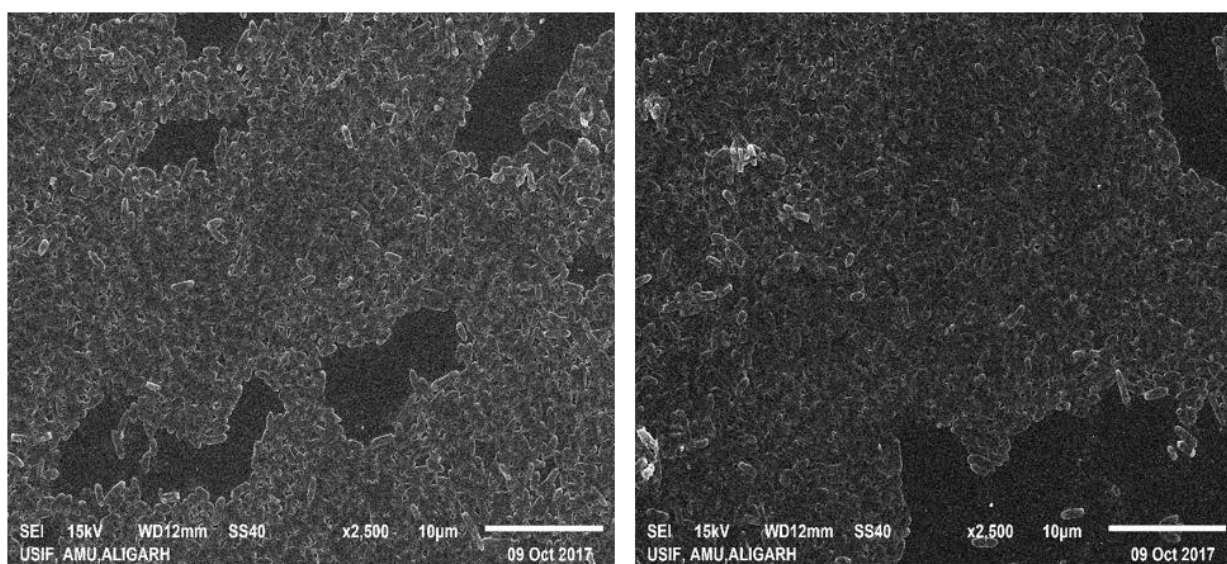
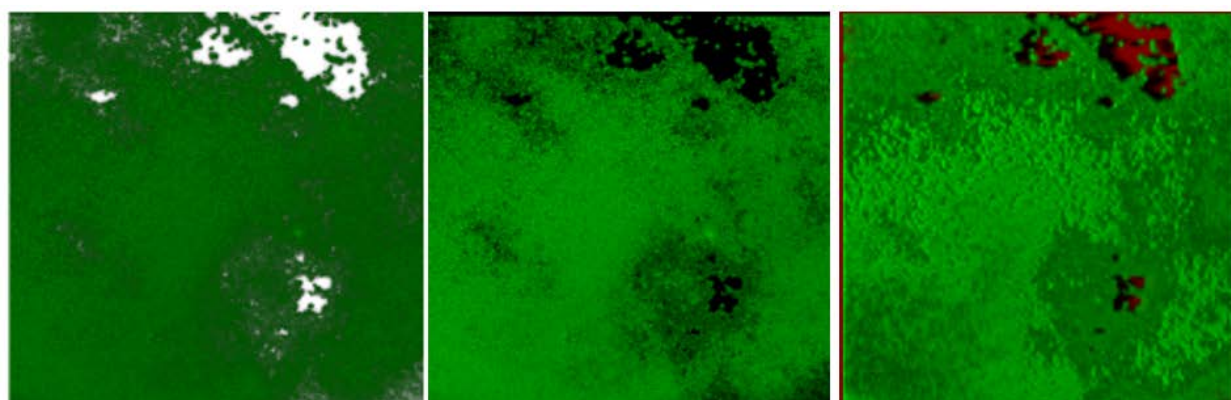
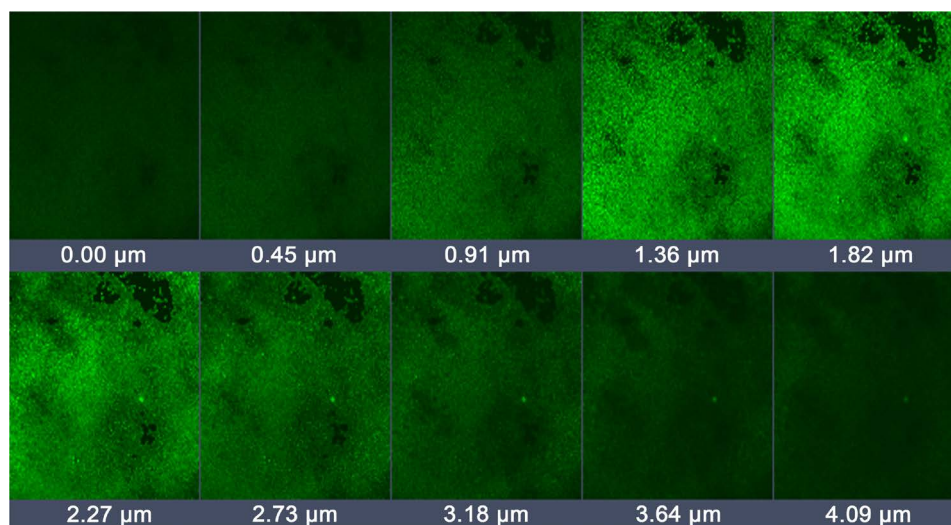


Figure 3. SEM images of biofilm development by *Pseudomonas entomophila* FAP1 on the glass cover slip.



(a)



(b)

Figure 4. (a) 3D confocal micrographs of mature biofilm formed by *Pseudomonas entomophila* on glass coverslip stained by 0.1% Acridine orange; (b) 3D confocal micrographs of mature biofilm with Z-stacks showing depth of biofilm at different stacks.

of *Pseudomonas entomophila* FAP1. It is evident from **Figure 5** that test strains attached to the root surfaces *in vitro*. It was observed that the strain FAP1 attached to the root surface in dense form at a different portion of the root surface and showed strong root colonization in the form of microcolonies/biofilm.

3.10. Rhizoplane and Rhizosphere Colonization

The results for the viable count of rhizoplane and rhizosphere during first to fifteen days after transplantation (DAT) were observed. The *Pseudomonas entomophila* FAP1 strain in the biofilm mode of life on the rhizoplane was maintained up to 15 days. After 24 h and 48 h after transplantation, there was drastically altered the structured of preformed biofilm on the root surface that was re-located from the rhizoplane to rhizosphere in the form of colony count. Initial bacterial count of *Pseudomonas entomophila* FAP1 strain was 7.5 Log CFU/gm of root but when we transplant into the soil system, preformed biofilm on wheat root surface was turned down and detached from rhizoplane from 7.5 Log “CFU/gm” of root to 6.1 Log “CFU/gm” of root **Figure 6**. We handled the experiment up to

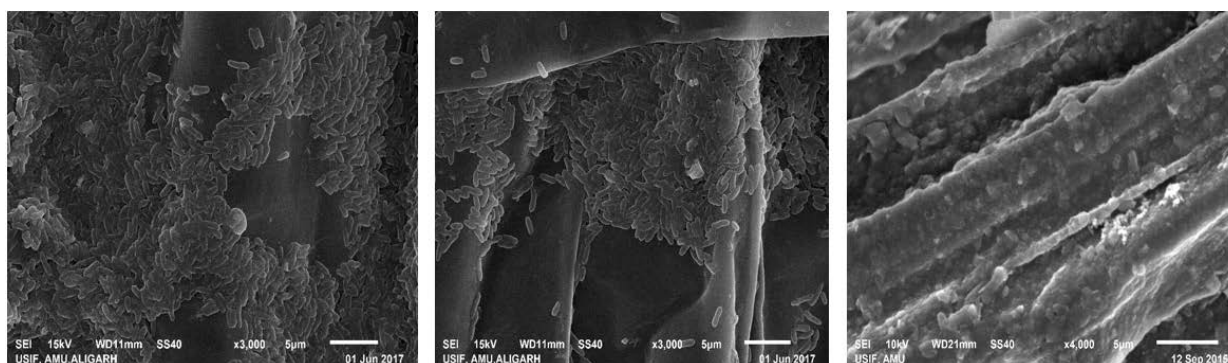


Figure 5. SEM images of biofilm development by *Pseudomonas entomophila* FAP1 on the root surface of wheat (*Triticum aestivum*) compared to uninoculated control.

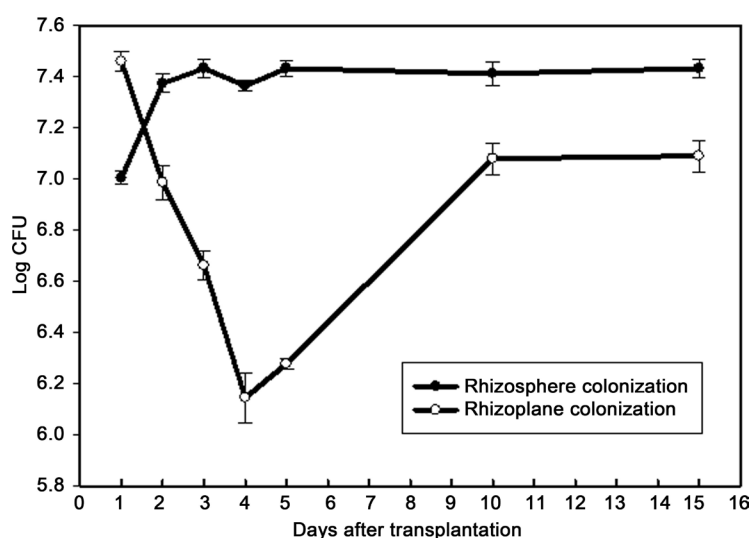


Figure 6. Rhizoplane and rhizosphere colonization by *Pseudomonas entomophila* FAP1 after different days of transplantation under soil microcosm.

15 days after transplantation and we found that after three days of transplantation, pre-formed biofilm on the rhizoplane was drastically switched over from the root surface to the rhizosphere as depicted in **Figure 6**. The strain *Pseudomonas entomophila* FAP1, after 5 days after transplantation there was reverse back to the rhizoplane from the rhizosphere. Rhizosphere colonization of the test strain exists in the significant number 7.5 Log “CFU/gm”, of soil in the rhizosphere up to 15 days after transplantation (DAT).

4. Discussion

Use of bioinoculant for promoting plant growth and protecting plant health is an integral part of sustainable crop production. Efficacy of bacterial inoculants on crop production depends upon the complex process of plant-microbe interaction and environmental factors [28]. Successful rhizosphere colonization by bacterial inoculants and expression of PGP traits including plant growth regulators is one of the major factors for plant growth promotion. Determination of the role of biofilm development by rhizobacteria in association with plants surface is the current topic under investigation [29] [30] [31] [32]. The intrinsic ability of bacteria to form biofilm *in vitro* and in association with plant root varied as reported by [33]. Understanding of rhizobacterial biofilm in the rhizosphere and their interaction to plant roots are still in the age of infancy. The natural interaction among the bacterial population and with plant roots in the natural soil system is predominantly in biofilm mode rather than a planktonic mode of lifestyle and these interactions may be positive or negative [2] [34] [35]. In this study, we have selected a *Pseudomonas entomophila* FAP1 strain after primary screening based on their multifarious PGP traits, tolerance to abiotic stress, biofilm development and biofilm-associated functions such as EPSs, alginate, swimming and swarming motility and cell surface hydrophobicity of the FAP1 has studied. *Pseudomonas entomophila* strain (FAP1) produced multifarious PGP traits, tolerate to abiotic stress and have the ability to form a strong biofilm. This combination of traits is expected to increase the plant growth under abiotic stress and protect the plant health by suppressing the pathogen. Further, the bacterium showed strong biofilm development ability *in vitro*.

Based on the above *in vitro* multifarious PGP traits, abiotic stress tolerance, biocontrol activity and biofilm development, we further investigated biofilm formation on a glass surface using 12 well tissue culture plates. This PGPR strain formed a strong biofilm. Such strong biofilm formation on the glass surface was demonstrated in other soil bacteria by [34]. We further investigated the behaviours of the FAP1 on ten days old wheat seedling root surface on the adherence and development of microcolonies/biofilm. The findings of root SEM analysis revealed that FAP1 strain adhered on the root surface and establish biofilm. However, this culture occupied root surface spatially separated as well as at a different portion of the root surface. [36] [37], observed that bacterial colonization and biofilm formation was important for the plant growth and protection

against phytopathogenic fungi in peanut plant. Our findings of biofilm formation on the wheat root surface by FAP1 are probably the first report on PGPR strain. In a separate experiment, we have transplanted the treated seedling of wheat by FAP1 isolate in sterile soil microcosm. The soil microcosm was maintained up to 15 days in climate-controlled growth chamber. Colonization of wheat rhizosphere as well as rhizoplane in treated seedling of wheat with FAP1 demonstrated its ability to survive, adjust and recolonize the rhizosphere/rhizoplane area with 15th days of transplantation of seedling in soil microcosm. A similar observation was revealed by [38]. However, PGPR are effective in colonizing the plant root and further multiply into microcolonies or produce biofilm as result of successful plant-microbe interaction and these plant associated biofilms are highly capable of providing protection from external stress, decreasing microbial competition and giving beneficial effects to the host plant supporting growth, yield and crop quality as reported by [39] [40] [41].

5. Conclusion

Based on above study it can be concluded that new isolate of PGPR with desired multifarious activity could be obtained through selective screening and biofilm development by such strain seems to be clear advantages for survival and rhizosphere colonization for enhanced plant growth. However, further experimentation is needed under natural soil system for practical exploitation in crop production/protection.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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