Exploiting Siderophore-Producing Rhizobacteria for Enhanced Iron Acquisition and Growth Promotion in Oryza sativa (L.) and Eleusine coracana (L.) Gaertn.

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Exploiting Siderophore-Producing Rhizobacteria for Enhanced Iron Acquisition and Growth Promotion in *Oryza sativa* (L.) and *Eleusine coracana* (L.) Gaertn.

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Abstract

Iron deficiency is a major nutritional constraint limiting plant growth and crop productivity in many agricultural soils. Plant growth-promoting rhizobacteria (PGPR) capable of producing siderophores can enhance iron availability and improve plant performance under iron-limiting conditions. In the present study, fifty rhizobacterial isolates obtained from diverse rhizosphere soils were screened for plant growth-promoting traits, including indole-3-acetic acid (IAA), ammonia, hydrogen cyanide (HCN), and siderophore production. Among the isolates, 36 exhibited siderophore-producing ability, and the most efficient strain was identified through 16S rRNA gene sequencing as *Pseudomonas fluorescens* MR8. Optimization studies revealed maximum siderophore production (82% siderophore units) in succinic acid medium after 72 h of incubation at pH 7.0 and 30°C. Spectroscopic characterization using UV-visible and FTIR analyses indicated that the siderophore possessed pyoverdine-like characteristics. The growth-promoting efficacy of *P. fluorescens* MR8 was evaluated in *Oryza sativa* (rice) and *Eleusine coracana* (finger millet) under iron-deficient conditions through in vitro co-cultivation and greenhouse experiments. Inoculation with the bacterial strain significantly enhanced seed germination, shoot and root growth, chlorophyll content, and iron accumulation compared with uninoculated controls. Rice exhibited a marked increase in lateral root development, while finger millet showed no significant change in root branching. Iron accumulation increased to 65.78 ppm and 81.0 ppm in rice and finger millet, respectively, under in vitro conditions, and further increased to 72.24 ppm and 95.3 ppm under greenhouse conditions. The findings demonstrate that siderophore-producing *P. fluorescens* MR8 effectively enhances iron acquisition and plant growth, highlighting its potential as a sustainable bioinoculant for improving crop productivity and nutritional quality in iron-deficient soils.

Keywords: Siderophore-producing PGPR, *Pseudomonas fluorescens* MR8, Iron acquisition, *Oryza sativa* (L.), *Eleusine coracana* (L.) Gaertn.

Introduction

Plant growth promoting Rhizobacteria (PGPR) is exerting direct or indirect beneficial effects on the growth and development of plants. These are free living root colonizing bacteria which either produces growth promoting compounds or suppress the growth of phytopathogens (Montano *et al*., 2014; Beneduzi *et al*., 2012). PGPR produces either auxins or auxins like compound, ammonia, phosphate

solubilizing enzymes, hydrogen cyanide and highly affinity ferric siderophore for the beneficial growth of the plants. In soil, the nutrients were not distributed homogenously, which is restricting to transport into the root for absorption. Therefore, an alteration in root architecture is needed to enhance the nutrient absorption (Lima *et al.,* 2010; Giehl *et al.,* 2012). Promotion of root growth by the PGPR bacteria is a good (index) marker which shows the effect of PGPR bacteria in plant growth and is characterized by rapid establishment of roots, by elongation for primary roots or by proliferation of lateral and adventitious roots, which is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment thus enhancing their chances of survival. It was recognized that most root promoting microbes synthesize IAA that their effect on plants mimics that of exogenous IAA (Glick, 2012). PGPR produces ammonia which induces lateral branches in roots thereby increasing the area of absorption of nutrients and minerals. PGPR are classified as biofertilizers, biocontrols- biopesticides and phytostimulants. Common PGPR identified were *Aztobacter*, *Pseudomonas fluoresens*, *Pseudomonas putida*, *Azospirillum*, and *Bacillus* etc., are characterized by their ability to promote plant growth, increase tolerance to environmental stress and/or enhance disease resistance (Montano *et al*., 2014; Dinesha *et al.,* 2015). Biocontrol mechanism of any PGPR can be due to production of HCN, Antibiotic, siderophore, enzymes or rhamnolipids (Goswami *et al*., 2014). Many studies demonstrated that PGPR bacterial siderophore are used by plants which improve nutrition in them by increases the Fe uptake and efficient biomass and photosynthetic metabolisms were enhanced.

Iron is one of the abundant microelements presents in the earth which is vital for cell metabolism, its scarcity leads to growth inhibition, decrease in DNA/RNA synthesis, alter in cell morphology, causes yield reduction and chlorosis in plants and anaemia in humans (Ahmed and Holmstrom, 2014; Nagoba and Vedpathak, 2011). It is assessed that approximately one third of earth can be measured as iron deficient (Radzki, 2013). It is a critical microelement required for the biosynthesis of chlorophyll moiety, act as a co-factor for several enzymes, structural component of leghaemoglobulin for symbiotic nitrogen fixation and also the component for ferritin (Sah and Singh, 2015; Pluhacek *et al*., 2016; Galet *et al*., 2015). The bio requisite of iron is very limited probably not greater than 10^{-17} M (Bholay *et al*., 2012). Despite its abundant in nature; it is unavailable to plant. In environment iron is in the form of a highly insoluble ferric oxides and hydroxide which is unavailable to microorganisms and plants (Galet *et al*., 2015; Braun *et al*., 2009). In ancient Greek siderophore mean iron carrier, for acquisition of iron from the nature certain rhizosphere microorganisms and grasses secretes an extracellular low molecular mass secondary metabolites (< 10 Kda) known as siderophore under iron deficient condition (Sah and

Singh, 2015; Ahmed and Holmstrom, 2014; Sayyed *et al*., 2010). Over 500s of bacterial siderophore have been defined chemically, were found to be synthesized by organisms belonging to *Zygomycetes, Streptomycetes, Enteriobacteriaceae, Pseudomonadaceae* families etc (Pluhacek *et al*., 2016). Microorganisms possess siderophore receptors that specifically recognize and take up their cognate siderophore iron complexes and retrieve in its system for their metabolic functions. The Primary role of siderophores is to scavenge Fe. They also form complexes with other essential elements (i.e. Mo, Mn, Co and Ni) in the environment and make them available for microbial cells (Ahmed and Holmstrom, 2014). Siderophores sometimes have roles not only in iron chelation, but can also have roles in other biological processes such as quorum sensing. In addition to metal transport through membrane, siderophore have antioxidant potential and antibiotic activity (Sah and Singh, 2015). The detection of siderophore can be done by Universal Chrome Azurol-S assay; bioassay and spectra assay (Schwyn and Neilands, 1987). Siderophore have wide application in the treatment of industrial waste, reprocessing of nuclear fuel and medication of metal contaminated sites (Sah and Singh, 2015). In recent research, siderophore would provide a good basis for transport of drugs through the bacterial membrane i.e., Trojan Horse Stratergy (Nagoba and Vedpathak, 2011; Pluchacek *et al.,* 2015). Furthermore, siderophores are able to reduce the oxidative stress by inhibiting the free radical formation and can inhibit the IAA destruction. Collectively they are able to reduce the plant stress factors and induce plant growth promotion.

Agricultural food production faces countless challenges due to increasing world population, climate change, rapid industrialization, urbanization and high use of classic pesticides indications to the alteration of fertile agricultural land to sterile due to the termination of beneficial microflora and fauna. Consequently, an alternative plant protection strategy are urgently required for sustainable agriculture and the biological control of plant diseases by beneficial microbes PGPR which offers significant potential for integrated plant disease management (Kong *et al*., 2016; Salmeron *et al*., 2016). Rice (*Oryza sativa*) is an important second largest crop cultivated worldwide and serves as a model crop in monocotyledon family. Rice is a deprived source of essential micronutrient i.e., Fe. Nutritive value iron (Fe) content was ~ 0.25 µg/g. Diet scarce in Fe in staple food crops causes 'hidden hunger' or micronutrient malnutrition in developing countries (Roy & Sharma, 2014).

Midst the crop plants, Finger millet (*Elusine coracana)* is a diverse group of small-seeded annual grass, have more stress-tolerant than the C3 crops, perhaps due to their antioxidant abilities attributed to withstand extreme environmental stress conditions and also needs least agricultural inputs. Finger millet is a choice for cultivation for poor and minimal lands in dry areas of temperate, subtropical and tropical

regions across the country. *E. coracana* cultivation constitutes ~12 % of the global millet area in Africa and South Asia including India and Nepal. It is rich in protein 7 to 14 % having nutritional values superior to that of rice and wheat. It is cultivated in India at a diverse range of eco-geographical locations under a variety of abiotic and biotic stress conditions such as drought, high temperature, high salinity, mineral deficiency, soil borne pathogen etc. (Venugopal *et al.,* 2016). Finger millet serves as a significant staple food for rural populations in tropical countries where calcium deficiency and anemia are wide spread due to Fe deficiency (Babu, 2007). Hence, due to malnutrition there is a requirement of nutrient rich staple crop which includes high Fe content in food crops which includes rice and finger millet. The PGPR and its secondary product siderophore may apply directly or indirectly for the growth enhancement and immobilization of minerals in plants. The present investigation is to isolate superior PGPR traits and16S rDNA sequence analysis for species identification. Further the PGPR were subjected to analysis the production and quantitative detection of auxin, ammonia and low molecular compound siderophore, HCN by PGPR traits and its direct influence of growth were studied on crop plants rice and finger millet.

Materials and Methods

Sample collection, Isolation and enumeration of bacterial population

Soil samples were collected from the rhizosphere of crop plants *viz., Arachis hypogea* (Ground nut)*, Zea mays* (Maize) and three medicinal plants *Solanum nigrum*, *Aloe barbadensis* (Aloevera) and *Bacopa monnieri* (Brahmi) grown in the MR, RZ, SR, MS and DP organic farms, Genwin Biotech PVT., Ltd, Tamil nadu, India. The soil samples were processed immediately for the isolation of microorganisms was done by serial dilution technique by using nutrient agar plates and incubated at 37^{o} C for 24 h. Once discrete well separated, morphologically differentiated and macroscopically visible 50 microbial colonies were picked up with the sterile inoculation loop and transferred to inoculums development media (Luria broth) and incubated at 37^{o} C in an orbital shaking incubator for 24 hrs. at 180 rpm and recognized as MR series (1, 2, 3 to 10), DP series (1, 2, 3 to 10), SR series (1, 2, 3 to 10), RZ series (1, 2, 3 to 10) and MS series (1, 2, 3 to 10).

Invitro screening and assessment of rhizobacteria for plant growth promoting activity

Detection of Indole acetic acid (IAA) production

Indole acetic acid production was quantitatively measured by the method developed by Goswami *et al*., 2013. Bacterial cultures were grown in a Luria-Bertani broth amended with 5 mM tryptophan for 3-4 days. Cultures were centrifuged at 10,000 rpm for 20 min. 2 ml of supernatant was mixed with 2 drops of ortho-phosphoric acid and 4 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% Perchloric

acid). Tubes were incubated at room temperature for 25 minutes. The intensity of pink color was read at 530 nm and the amount of IAA produced was extrapolated from the standard curve (100 µg/ ml standard stock solution of IAA in 50% ethanol).

Detection and assay for ammonia production

All the isolates were tested for the production of ammonia in peptone water containing (per liter) peptone 10 g, NaCl 5 g, yeast extract 5 g, and pH adjusted to 7.6. Freshly grown cultures were inoculated into 10 ml peptone water in each tube and incubated for 48 h at 28°C ± 2°C. After incubation the broth was centrifuged. Nessler's reagent (0.5 ml) was added to supernatant obtained after centrifugation of the broth. Development of brown to yellow color is a positive test for ammonia production (Ahmad *et al*., 2008) and its optical density was measured at 450 nm using UV- Visible spectrophotometer. The color changes were compared with calibrated standards of 0.5 µmol, 1 µmol, 2.5 µmol, 5 µmol, 10 µmol and 50 µmol ammonia solutions.

Detection of HCN production

All the 50 isolates were screened for the HCN production accordance with Ahmed *et al.,* 2008; Goswami *et al.,* 2014. Nutrient agar plates were prepared with 4.4g/L glycine and streaked with isolated bacterial culture. A watman No.1 filter paper was presoaked in 2% sodium carbonate in 0.5% picric acid solution. The filter paper was placed in the upper lid of the plates and sealed with parafilm. The plates were incubated at 28±2ºC for 4 days. Orange to red or brown color development indicates HCN production.

Siderophore production and detection assessment

Siderophore are only produced under iron limited condition, the isolated strains were grown in media devoid of iron. Minimal media 9 (MM9) was used to check the growth and siderophore production for 48 h on a rotary shaker at room temperature. After 48 h of growth, the culture was centrifuged at 13,500 rpm for 2 min and the supernatant was separated and stored at 4º C. All glassware's were used to store the stock solution of MM9 medium was treated with 6N HCL to remove trace amount of contaminating iron and the glassware were rinsed thoroughly with double distilled water. Preparation and detection were done according to Schwyn and Neilands, 1987 modified; Mikicinski *et al*., 2016; Louden *et al*., 2011. To prepare 1 L blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1mM $FeCl_3.6H_2O$ dissolved in 10 mM HCl). 72.9 mg HDTMA (CTAB) dissolved in 40 ml water were slowly added under stirring condition. The resultant dark blue liquid was added with 750 ml water, 100X MM9 salts, 15 g agar, and 30.24 g pipes. The pH of the solution is maintained at 6.8 and autoclaved. After cooling to 50º C, 30 ml of casamino acids (10%) were slowly added. The CAS plates were prepared and each plate contains 30 ml of blue agar. Using a sterile cork borer 2-4 fine wells were

made on the blue agar plate. Culture supernatant was added to the well (60 µL), and the plate was incubated at 37° C for 48 h to develop colour change. If siderophore is present, an orange halo is visible around the well it indicates positive result.

Siderophore production assay were quantitatively quantified using CAS shuttle assay (Payne, 1994; Goswami *et al.,* 2014; Sayyed and Chincholkar, 2010). A 6ml 10 mM HDTMA solution was places in a 100 ml volumetric flask and diluted with water followed by 1.5 ml iron (III) solution and 7.5 ml of 2 mM CAS solution was slowly added under stirring. A 4.3 g of piperzine was dissolved in water and 6.25 ml of 12 M HCl was added carefully. This buffer solution (pH 5.6) was rinsed into volumetric flask which was then filled with water to afford 100 ml of CAS Assay solution. 1 ml (48 hrs.) supernatant siderophore solution was mixed with 1ml of CAS Assay solution and incubated for 1 hour. The absorbance was measured at 630 nm using UV-Visible spectrophotometer. Percent de-colorization (Siderophore Units) was calculated by using following formula

$$\text{Siderophore Units (\% decolorization)} = \frac{(Ar - As)}{Ar} \times 100$$

Where Ar = Absorbance of reference at 630 nm and As = Absorbance of sample at 630 nm.

Biochemical characterization and 16S rDNA ribotyping analyses of PGPR bacteria

The positive and the superior PGPR bacteria have the ability to produce auxins, ammonia, HCN and siderophore microbial culture MR 8 was subjected to biochemical characterization for genus identification using Bergey's Manual of Systematic Bacteriology (Bergey *et al*., 1990; Holt *et al.,* 1994). The genomic DNA isolated and purified by using DNA isolation and purification kit (Chromos Biotech, Bangalore). The DNA was quantitatively determined at 260 nm. The the good quality of isolated DNA was analysied for 16S rDNA gene sequences. The DNA was amplified with universal primers standard universal forward primer 5'- GTTTGATCCTGGCTCAG-3'and standard universal backward primer 5'- ACGGGCGGTGTGTNC- 3' (Gaonkar *et al.,* 2012; Nakouti *et al.,* 2012). Reaction mixture and thermocycling condition was setup accordance with Dinesha *et al.,* 2015. The PCR products were sent to Bioserve, Bangalore, India. According to their requirements the sequence was done. The derived 16S rDNA gene sequence was compared with sequences in GenBank using BLAST program.

Culture condition and medium optimization for the production of siderophore

Siderophore Production in Different Media

For the production of siderophores, Cas-amino acid medium (CAM), Nutrient broth (NB), Succinic acid medium (SM) and Fiss-glucose minimal media (FGMM) and King B medium (KB) were separately

inoculated with MR8 culture and incubated for 48 hrs. The supernatant contents were estimated quantitatively by CAS assay at 630 nm.

Effect of pH on siderophore production

The MR8 culture stains were grown in 100 ml Succinic acid media due to the increased production of siderophore. The culture was grown at different pH (4, 5, 6, 7, 8, 9 and 10). After 48 h, the supernatant content was measured spectrophotometrically at 630 nm and siderophore production was estimated quantitatively.

Effect of temperature on siderophore production

The MR8 culture stains were grown in 100 ml Succinic acid media. The culture flasks were grown at different temperatures (25°C, 30°C, 35°C, 37°C and 40°C). After 48 h, the supernatant content was measured spectrophotometrically at 630 nm and siderophore production was estimated quantitatively.

Effect of duration of incubation on siderophore production

The isolated culture strain was grown in SM medium in Erlenmeyer flask for 5 days on a rotary shaker at room temperature. After interval of every 24 h of growth, the culture was centrifuged at 13,500 rpm for 2 min and the cell supernatant was subjected to spectrophotometric assay at 630 nm until 5 days.

Mass production of siderophore using fermentor

After optimization of culture conditions pH, temperature, time duration and medium an enhanced production of siderophore were carried by using fermentor. The experiment was carried out in a 3 L of glass fermentor (Applikon Bioconsole ADI 1025 model). The glass fermentor were first washed thoroughly and rinsed with 6N HCl to remove trace metals present previously and the glass vessel were washed with distilled water. The glass fermentor was filled with 1.5 L of SM medium and sterilized at 15 lbs for 15 minutes using autoclave. The batch culture with a working volume of 1.5 L was run under constant limitations at 30º C, pH 7.0 and 200 rpm. The gas flow allowed the exchange of 1 volume of air per volume of medium per minute. The pH was maintained constant by automatic titration with 1N NaOH or 1N HCl and the temperature was automatically maintained by inlet, out let flow of water. The culture inoculums MR8 of 5% was suspended in 5ml sterile distilled water and with the help of inoculation syringe and transferred the cell suspension aseptically into the glass fermentor. Batch fermentation was carried out for 96 h at all the parameters are kept constant. After 24 hrs interval the samples were collected aseptically and subjected to determine the % siderophore unit the broth using spectrophotometric assay at 630 nm and every 12 hours of incubation, the culture was checked for the presence of contamination by staining process. These were repeated until 96 h and tabulated the reading.

Extraction and Purification of Siderophore (Tailor and Joshi, 2012)

The siderophore containing fermented solution was taken and centrifuged at 13,500 rpm for 5 minutes. The supernatant was taken and adjusted to pH 6.0 and subjected to ammonium sulphate precipitation in ice cold condition with 85% saturation for 30 minutes. The contents were centrifuged at 10,000 rpm for 10 minutes. The pellets formed were dispended in 100ml of 100mM Tris buffer. The the centrifuged contents were poured into activated dialysis bag and the extract was exhaustively dialyzed against 0.3 mM phosphate buffer (pH 7.0). Dialysis was carried out for overnight at cold condition the buffer was changed for every two hours to remove ammonium salts. The overnight dialyzed siderophore content was subjected to solvent extraction. The siderophore content was acidified to pH 4.0 using 12 M HCl and extracted with equal volume of ethyl acetate. Ethyl acetate fractions were pooled together and concentrated by using rotary vacuum evaporator. Dried fractions were then resuspended in methanol (1 mg/ml) and subjected to further purification. Siderophore purification was done by Column Chromatography Sephadex LH-20 column was prepared by mixing 50 g sephadex in methanol with gentle shaking for 20 min, and subsequently, material was packed into a 50×1.5 cm column without any air bubbles and equilibrated with four bed volumes of methanol. The concentrated siderophore sample was then loaded on column and eluted with methanol with flow rate of 1 ml/min. Fractions of 5 ml each were collected and quantified for their siderophore units using CAS assay. The methanolic fractions positive for siderophore units were pooled together and evaporated to concentrate using a rotary vacuum evaporator and finally dried sample was stored at –4°C.

UV Spectral analysis and Fourier-transformed infrared (FTIR) spectroscopic analysis of bacterial siderophore

The obtained siderophore is spectrophotometrically analyzed for UV activities for maximum absorption and FTIR spectroscopic analysis of siderophore was recorded in the region 400-4000 cm-1 using SHIMADZU FTIR, Japan. The dried siderophore obtained were subjected to FTIR analysis and the peaks obtained were compared with the standards. The spectral analysis was done in accordance with Tank *et al.,* 2012.

Co-cultivation of Plants and Bacteria

Invitro co-cultivation of PGPR bacteria with rice and finger millet

The healthy fertile *Oryza sativa* (Co. 47 rice variety) and *Eleusine coracana* (Co. (Ra) 14 finger millet variety) seeds were obtained from Regional Research Station, Tamil Nadu Agricultural University, Paiyur, Tamil Nadu, India. The seeds were washed with running tap water (1 hrs.) followed by surface sterilized with 0.1% mercuric chloride and 70% ethanol for 1 min. The surface sterilized seeds were inoculated in co-cultivation plates. The co-cultivation media plates were prepared by pouring the plates

half with SM agar medium for the enhanced production of siderophore by PGPR and another half with half strength MS medium for seed germination. In the Succinic medium one set of plates was carried with the superior trait PGPR was inoculated by streaking adjacent to the SM agar, care should be taken that the inoculum should not touch the half strength MS medium known as bipartite petridish (Weise *et al.,* 2013). Another set of plates was added with 1 ml fermented siderophore broth obtained in the above procedure in the pre made wells. Place a glass rod beneath the plates make that the SM agar should be at the top because, the siderophore and other growth factor produced by the PGPR should diffused into the half strength MS medium which was utilized by the plant growth. The plates were kept under controlled environmental condition 25±2ºC, 4000 lux light intensity and 70-80% humidity (Esseminea *et al.,* 2017). The effect of PGPR bacteria on the percent seed germination, shoot length, root length and total plant length, establishment of roots and adventitious branches were observed between uninoculated controls and treated.

Co-cultivation and growth of seeding in soil experiment

Direct co-cultivation study was carried out by soil experiment in triplicates in the polytray 18 X 13 cm in the month of January where the average temperature was 28±3ºC. Rice and finger millet were surface sterilized and incubated in PGPR bacterial culture (2 x 10^{11} CFU) and siderophore unit solution (82%) for 1hrs following the method of Omidvari *et al.,* (2010). The seeds were sown in polytray containing 17% H_2SO_4 acid washed sterile soil to remove iron contamination followed by autoclaving for one hour on alternate three days to eliminate soil borne microorganisms (Sharma & Johri, 2003). The percent seed germination, total plant length, shoot length, root length, establishment of roots and adventitious branches parameters were analyzed between controls PGPR treated and siderophore treated. Autoclaved double distil water were used to spray for growth period periodically. The seed inoculated tray was placed in a green house at 25±2ºC. The results were obtained after 10 days of inoculation.

Estimation of pigments and protein

Chlorophyll estimation

The chlorophyll estimation was done in accordance to Sadasivam and Manickam, 1996. 1g of surface sterilized leaf sample were grind to a fine pulp with 20 ml of 80% acetone centrifuged at 5000 rpm for 5 min and transferred the supernatant to a 100ml volumetric flask. Repeat this until the residue is colorless and bring the final volume to 100 ml with 80% acetone and read the absorbance at 645nm, 663nm and 652nm. The amount of chlorophyll was calculated using the equation.

$$\text{Chlorophyll a mg/ g tissue} = 12.7\ (A_{663}) - 2.69\ (A_{645}) \times \frac{V}{1000 \times W}$$

318 Chlorophyll b mg/ g tissue = 22.9 (A_{645}) – 4.68 (A_{663}) x $\frac{V}{1000 \times W}$

320 Total Chlorophyll mg/ g tissue = 20.2 (A_{645}) + 8.02 (A_{663}) x $\frac{V}{1000 \times W}$

322 A= absorbance at given wavelength, V= final volume of extract, W= fresh weight of the tissue.

323 **Fe content determination by atomic absorption spectroscopy**

324 0.5 g of fresh leaf samples were weighed and treated with concentrated HNO_3 and $HCLO_4$ (10:4). The
325 digested sample was closed and heated in microwave oven. The heated samples were allowed to cool at
326 room temperature and filtered by Whatman No. 1 filter paper into a 25 mL in volumetric flask and make
327 the volume using double distilled deionized water. These extracts were used for the determination of Fe
328 through AAS (Jajda *et al.,* 2015).

329 **Statistical analyses**

330 Analysis was carried out using triplicate value as mean ± standard deviation, $P < 0.05$ to identify
331 significant difference in each parameter between controls and treated with PGPR on rice and finger
332 millet.

333 **Results**

334 **Invitro screening of bacterial isolates for their plant growth promoting activities**

335 **Screening and estimation for Indole Acetic Acid production**

336 Production of auxin or growth promoting component is one of the important traits of the PGPR bacteria.
337 All the 50 isolates were grown in LB broth supplemented with tryptophan for 3 days because tryptophan
338 is the precursor amino acid utilized for the synthesis of IAA by plants and microbes. It was found that
339 12 isolates were positive for the IAA production by the appearance of pale pink color and without
340 tryptophan IAA production was not observed and the details of the PGPR isolates produces IAA were
341 given in the **Fig. 1.** The concentration of IAA in the medium was estimated. The maximum IAA was
342 produced by PGPR bacteria DP 6 strain (38.6 µg/ml), MR 2 strain (36.2 µg/ml), MR 8 (34.8 µg/ml) and
343 the lowest was produced in the strain MS 7 (8.0 µg/ml).

344 **Screening for ammonia production**

345 The presence of ammonia was examined in all the 50 bacterial isolates which was characterized by the
346 change of colour from brown to yellow and it was found that 36 isolates showed colour changes and the
347 concentration was estimated by Nesslers reagent. The concentration of the ammonia was calculated by
348 plotting the OD value in a standard graph. Ammonia produced by the isolates was depicted in **Fig. 2**.

Maximum production of ammonia was estimated in the MR 8 trait (5.64 µmol/ml), DP 8 (4.9 µmol/ml) and the least ammonia concentration was produced by RZ 6 (0.9 µmol/ml).

Detection of HCN production

All the 50 isolates were subjects to the detection of HCN production. However, only 4.5% was detected as an excellent HCN producer by observing the color change from orange to brown and 11% of the isolates were poor HCN producer, the residual isolates were not able to detect the HCN production due to the color change in the plates neither were nor observed. Bacterial trait MR 8 and DP6 were recognized as superior HCN producers.

Detection and screening for siderophore production

All the 50 isolates were preliminary screened for the production of siderophore using CAS agar plates. The isolates were grown in iron limited MM9 medium for 48 h and the culture was centrifuged, the supernatant solution of 60 µl was added to the wells made in the CAS agar plates. After the proper incubation of 48 h an orange halo zone were produced around the supernatant containing the siderophore, which indicates the positive trait for siderophore production and the absence of colour or halo zone determine negative trait for siderophore production. The diameter of the zones was measured and the largest siderophore zone was observed in the media with MR 8 (20.8 mm) shown in Fig. 3 followed by DP 6 (19.5 mm) and the smallest zone in diameter was detected in the trait MR 7 (6.6 mm). Out of 50 isolates 8 organisms were preliminary screened as an excellent siderophore producer and 21 other trait was able to produce siderophore in low level. The results were given in **table 1 and fig.3.**

All the 29 preliminaries positive siderophore producers determined by CAS agar plate methods were further preceded for the quantitative assay by CAS shuttle spectrophotometer assay at 630 nm. Subsequent addition of culture supernatant to the CAS assay solution showed decolourization of the deep blue colour and yellow, golden yellow and deep orange color was developed. The siderophore unit produced was estimated and the maximum of siderophore units was produced in the MR8 trait (60.2%), DP6 (54.7%), SR8 (51%) and the least siderophore units was estimated in MR7 (10.5%). The quantitative data were given in Fig. 4 and fig.5.

Biochemical characterization and 16S rDNA sequence analysis of PGPR bacteria MR8.

Based on the above quantitative estimation of IAA, ammonia and siderophore production the superior PGP trait MR 8 (Fig. 6.) was used for further study i.e., strain identification and *invitro* effect of PGPR MR 8 traits on co-culture. The PGPR bacteria MR 8 were gram negative, rod shaped and positive for motility test, indole test, methyl red, citrate utilization test, gelatin hydrolysis test, catalase test, oxidase

test, and pigment production. Negative for sporulation and VP test. In accordance with Bergy's manual the PGPR MR8 were identified as *Pseudomonas* sp. 16S rRNA gene analysis confirmed that the isolate was Pseudomonas fluoresens (The GenBank accession no. KY190964, *Pseudomonas fluorescens* strain MR8). The phylogeny was computed in the Fig.7. The blast analysis showed 99% similarity with 16S rDNA sequence of *P. fluorescens.*

Media optimization and culture condition for the enhanced production of Siderophore by *Pseudomonas fluorescens* strain MR8.

The best siderophore producing PGPR *Pseudomonas fluorescens* MR 8 was inoculated in five different medium and confirmed for the increased production of siderophore by spectrophometrically at 630 nm. The highest siderophore units was produced in succinic medium (72.0%) followed by cas-amino acid medium (62.8%), glucose medium (66.5%), KB medium (59.6%) and the least siderophore units obtained in NB medium (29.9%). PGP trait Pseudomonas sp. MR 8 was cultured in succinic medium having different pH 4-10 (Buffer phosphate). The microbial growth was relatively very poor in acidic pH 4.0 and basic pH 9, 10. Due to this pH effect the siderophore unit produced was relatively less in pH 4, 9 and 10. The enhanced production of siderophore units was determined between pH 6- 8. At neutral pH the highest siderophore units was quantitatively estimated (73.2%). PGP trait Pseudomonas sp. MR 8 was cultured in medium with neutral pH and incubated at various temperatures. The microbial growth was relatively very poor in high temperature 40^{o} C (15.6%). At 30^{o} C the greater production of siderophore units was estimated as 71.2%. Siderophore production was increased after 48 h of incubation SU's (71%), conversely the maximun production of siderophore units was assessed at 72 h (76%). After 96 h incubation there is a drastic fall of siderophore production were estimated i.e., SU (56%). This might be reabsorption of siderophore by the PGP trait. The results were depicted in Fig.8.

Mass production of siderophore in fermentor

The production of siderophore was carried for 96 hrs in fermentor with all the parameters kept constant and it was found that the siderophore production was credentials increased from the 24 hrs of incubation. The siderophore units produced during 72 hrs was quantitatively estimated found to be the highest (82%). After 72 hrs of fermentation there is a drastic decrease of siderophore and after 96 hrs the amount of siderophore was found to be 61%. The production of siderophore was found to be maximum at 72 hrs and this was observed by colour change in the fermentor shown in Fig. 9.

Siderophore purification.

Column chromatography was performed and 25 fractions was eluted using methanol of 5 ml each. The orange tinge fraction was detected as siderophore positive using CAS assay, all the orange tinge fraction

were pooled together and concentrated using rotary evaporator and the golden yellow powder of siderophore was stored at – 4ºC.

UV & FTIR spectral analysis

A UV spectrophotophometric analysis of siderophore showed an absorption with a sharp peak indicates the siderophore is active in UV. Chemical characterization of siderophore functional group was determination through FTIR spectrum showed a peak at 3334.94 cm-1, indicating the presence of a primary alcohol group. A strong peak at 1637.89 cm-1 shows the presence of C–OH stretching in the purified compound. A band at, 2172.44 was recorded indicates the existence of C-N Fig.10. The results of this study are in accordance with Tank et al., 2012; Davidson et al., 2003.

Effect of PGPR *Pseudomonas fluorescens* MR8 and its Siderophore with rice and finger millet.

Invitro co-cultivation of *Pseudomonas flurosecens* MR 8 with rice and finger millet

Under aseptic, controlled light intensity, temperature and humidity the *Oryza Sativa* and *Elusine coracona* seeds were inoculated in the plates specially designed for co-cultivation and checked for growth after 10 days of inoculation. Summary of the invitro screening is shown in Table.3. Rice and finger millet with 2 x 10^{11} CFU by co-cultivation with *P. fluorescens* MR8 shows a significant increase in percent germination treated with PGPR *P. fluorescens*. Similarly, the shoot length, root length was also increased with the production auxin and greater stimulatory effect on lateral root might be due to the production of ammonia by the PGPR (Fig.11).

Invivo co-cultivation of *Pseudomonas flurosecens* MR 8 with rice and finger millet

The surface sterilized *Oryza Sativa* and *Elusine coracona* seeds were inoculated in sterilized soil tray for co-cultivation with PGPR and siderophore. The growth was recorded after 10 days of inoculation. Summary of the invivo experiment was shown in Table 2 and Table.3. Rice and finger millet with 2 x 10^{11} CFU by co-cultivation with P. fluroscens MR8 shows a significant increase in percent germination treated with PGPR *P. fluorescens*. Similarly, the shoot length, root length was also increased with the production auxin and greater stimulatory effect on lateral root might be due to the production of ammonia by the PGPR (Fig.12).

Discussion

PGPR are beneficial microbes well known mechanisms for the production of IAA, HCN, siderophore, ammonia often act as biological control by the production of antibiotics and also increases nutrient uptake by the plant (Dominik *et al.,* 2016). In the present study, about 50 rhizobacteria were isolated from different ecological place and screened invitro for plant growth promoting activities. Similar study

of preliminary isolation work done to isolate PGPR from soil environment by Dinesh *et al.,* 2015; in high salinity and high acidic pH soil (Goswami *et al.,* 2014). Out of total 50 isolates 12 could produces IAA when supplemented with tryptophan. IAA allows plants to develop an organized root system for nutrient uptake. Similar to the present study Ahmad *et al.,* 2008; Goswami *et al.,* 2014 reported that the enhanced production of IAA was done with additional supplements of tryptophan in the medium. The ammonia production was estimated maximum in the PGP strain MR8 of 5.6 µmol/ml. Ammonia productions is an additional N source in soil by the rhizobacteria. The greater accumulation of ammonia level higher than 0.1mM, in the plant cells inhibits the seed germination and seedling establishment. Ammonia can also stimulate root branching (Kisiel and Kepczynska, 2016). Similar trend of ammonia production was also reported by Bharucha *et al.,* 2013; Dinesh *et al.,* 2015. HCN detection is one of the antimicrobial activities of the PGP trait, PGP trait MR8 were recognized as superior HCN producer (Premachandra *et al.,* 2016).

The detection and screening of siderophore production result revealed that 29 isolates were determined as positive siderophore producer using CAS plate assay and CAS shuttle assay at 630 nm. The maximum siderophore zone was observed in PGP trait MR8 of 20.8 mm and 60.2% siderophore units were estimated (Radzki *et al.,* 2013; Gamit and Tank, 2014; Ahmad *et al.,* 2008; Goswami *et al.,* 2014). Based on the above discussed PGP activities like IAA, ammonia, HCN and siderophore production PGP trait MR8 is found to be superior. The 16 S rRNA gene analysis and the phylogenetic tree showed 100 % similarities with *Pseudomonas fluoresens* (Kisiel and Kepczynska, 2016; Embaby *et al.,* 2016; Sharma and Johri, 2003). In succinic acid medium the maximum siderophore units were produced (72%) among other media i.e. Cas- Amino acid, Glucose, King B and Nutrient broth. Our result reveals the production of siderophore was greatest in neutral pH, 30^{o}C at 72 hrs of incubation. Sridevi *et al.,* 2008 obtained maximum siderophore production at 24 hrs of incubation in Rhizobium sp.; Sayyed *et al.,* 2010 also reported the maximum siderophore yield in *P. fluorescens* 5096 in SM at 24 hrs of incubation. The present finding is partially in accordance with Embaby *et al.,* 2016 successfully produced siderophore from 24 h to 48 h. Maximum yield of siderophore is a crucial factor and profitable bioprocess, in this regard the physicochemical factors kept constant for the production of siderophore using fermentor. The maximum siderophore units obtained during 72 h was estimated to be 82 %. Similar to our present work maximum 92% siderophoe units was obtained in Alcaligenes faecalis during 30-36 h in alkaline condition (Sayyeed *et al.,* 2010). The dried partially purified siderophore was subjected to UV & FTIR spectral analyais, based on the obtained data the siderophore is recognized as pyoverdine type which is partially in accordance with Tank *et al.,* 2012; Davidson *et al.,* 2003.

Plants require an essential element Fe for its cycle. Hence deficiency leads to an economic impact on reduced yield and quantity. To overcome this problem synthetic chelates like EDTA and EDDHA are widely used, however it is poorly biodegradable and causes treat to the environment and finally accumulate in ground water (Radzki *et al.,* 2013). Our results identified *P. fluorescens* MR8 efficiently increases plant growth and nutrient impart i.e. Fe content in rice and finger millet both invitro and invivo experimental design. The present work reveals a wide stimulatory effect of PGPR treated and siderophore treated vegetative growth in percent germination of seeds, shoot length and root length in both rice and finger millet in bipartite invitro co-cultivation and also in vivo pot cultivation. However, the no. of adventitious root was significantly increased only in rice and not determined in finger millet. Related to our report Goswami *et al.,* 2014 has reported the growth promotion in *Arachis hypogea* by PGPR has enhanced plant growth under saline desert condition. Montano *et al.,* 2014 also described PGPR promote similar growth in maize, wheat, rice soyabean and bean. Castro et al., 2013 evaluated the activities of PGPR using co cultivation with Arabidopsis and given strategy for PGPR identification. Similarly, Badawi *et al.,* 2011 tested PGPR *Bradhyrhizobium* and some rhizo bacteria can increases the vegetative growth in peanut plants.

Plant pigments chlorophyll a, chlorophyll b and total chlorophyll of leaves increased after 10 days of inoculation of PGPR and siderophore in rice and finger millet. In rice siderophore treated plants was determined the maximum chlorophyll contents to that of PGPR treated and control treated. Vice-versa results was obtained in finger millet, the increased chlorophyll content was recorded in PGPR treated to that of siderophore treated and control treated. Similar to the recent work P. fluorescens UM 270 promoting growth in Medicago tanculata plants by increase in biomass and chlorophyll content (Salmeron *et al.,* 2016). Likewise increase in chlorophyll content was determined in tomato plants using bacterial siderophore under hydrophonics culture condition (Radzki *et al.,* 2013).

The present plant growth experiments suggest increase Fe content was determined using AAS after 10 days of both invitro and invivo incubated of seedlings treated with PGPR P. fluorescens MR8 and siderophore when compare to the control seedlings both rice and finger millet. Related to the present work Sah *et al.,* 2017 reported the increases Fe content in stem, leaf and seeds of maize plant. Jin *et al.,* 2010 also determined that siderophore secreting microbes turn help to improve the elevation in Fe uptake by red clover plant.

Conclusion

This study highlights the importance of isolation, selection and evaluation of PGPR bacteria. *Pseudomonas fluorescens* MR8 produces enchanced production of IAA, HCN, Ammonia and

siderophore. PGPR microbes in favoring more siderophore secretions under Fe- deficient conditions in succinic acid medium, which helps to solubilize the insoluble Fe to soluble forms. We believed that the solubilize Fe was taken up by the plants through root adsorption and play a very important role in Fe acquisition and nutrient impart in plants. We conclude that the direct application of PGPR *Pseudomonas fluorescens* and its siderophore pyoverdine stimulates increases in percent germination, shoot length, root length, chlorophyll content, Fe ion acquisition in *Oryza sativa* and *Eleusine coracana* and also we concluded the increased number of lateral root branching for more anchorage to soil for its greater establishment and more nutrient uptake for adaptive functions in *Oryza sativa* and *Eleusine coracana*. Therefore, siderophore producing *P. fluorescens* hold high promising as an alternative nutrient management for sustainable cultivation of agro crops.

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Contributions

Shiv Shankar Chinnasamy and Krishnagowdu Saravanan, Conceptualization and Idea; Shiv Shankar Chinnasamy performed the experimental work with the assistance of Sathish Sekar Dhanasekeran; preparation of the tables and figures: Sundarasamy Dhanapal, Ravi Velusamy, and Elangovan Namasivayam; Shiv Shankar Chinnasamy wrote the initial draft; Krishnagowdu Saravanan corrected and revised the manuscript; finally, authors read and approved the manuscript.

Ethics declarations

Competing interests

The authors declare no competing interests.

Data Availability Statement

The data sets were generated during the experiments has been mentioned as tables and figures.

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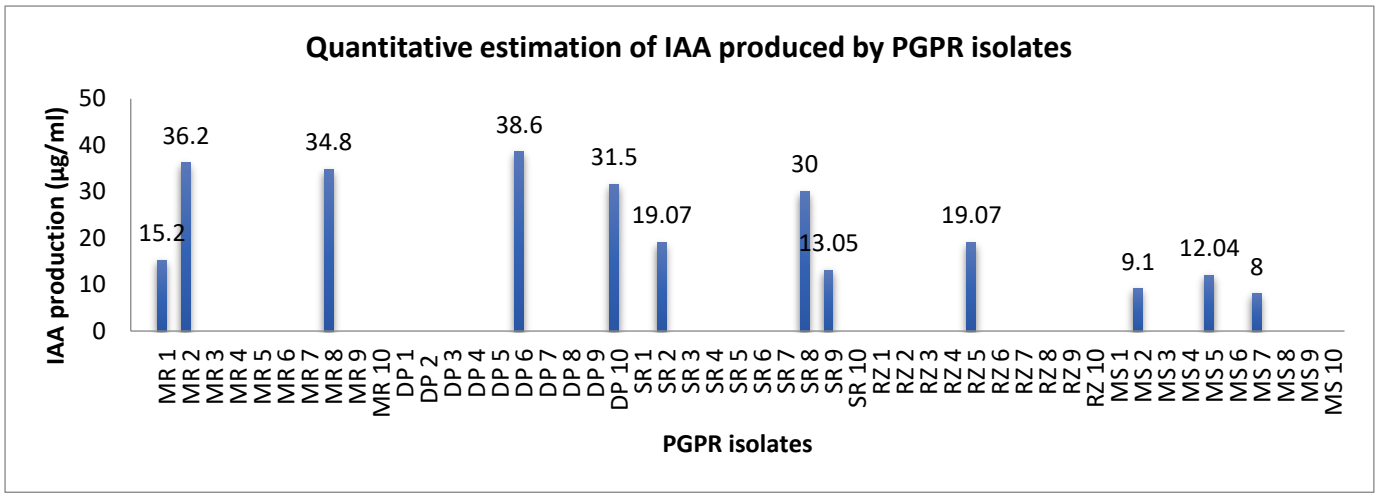


Fig.1. Quantitative estimation of IAA production by PGPR isolates.

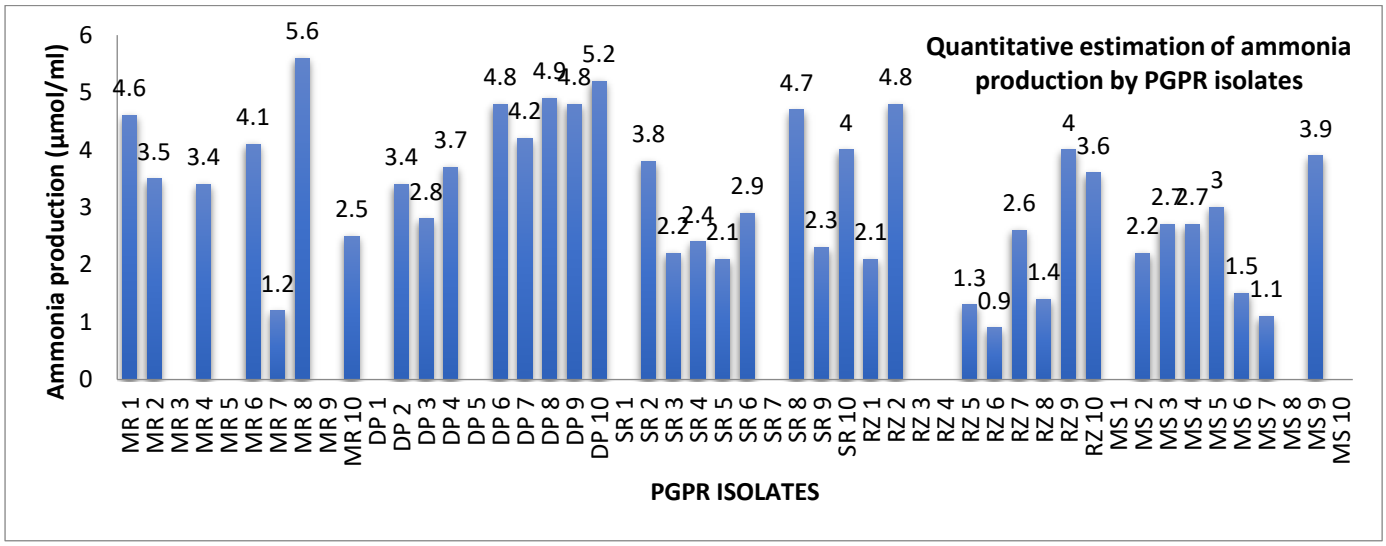


Fig.2. Quantitative estimation of ammonia production by PGPR isolates.

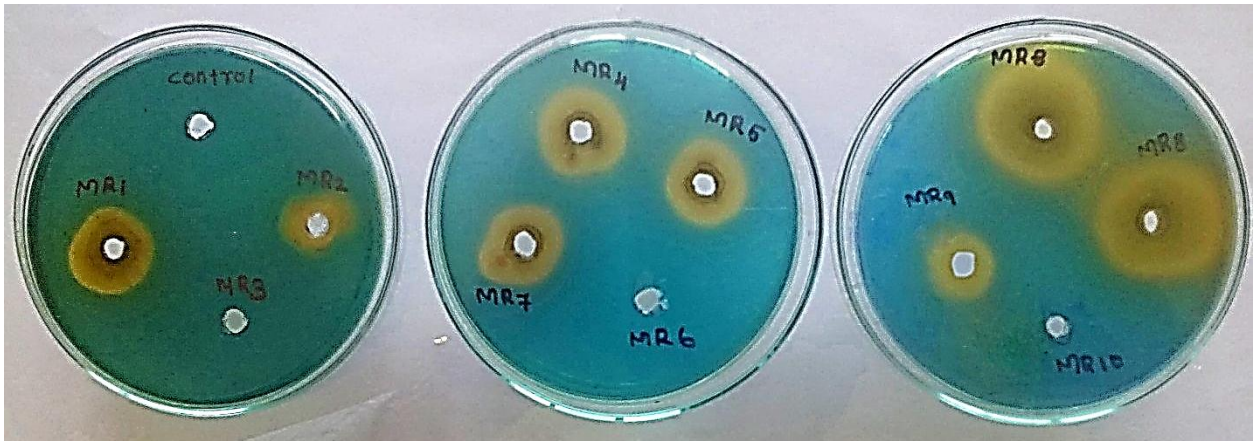


Fig.3. Orange halo around the wells in blue agar showing siderophore production by MR series after 48 h of incubation

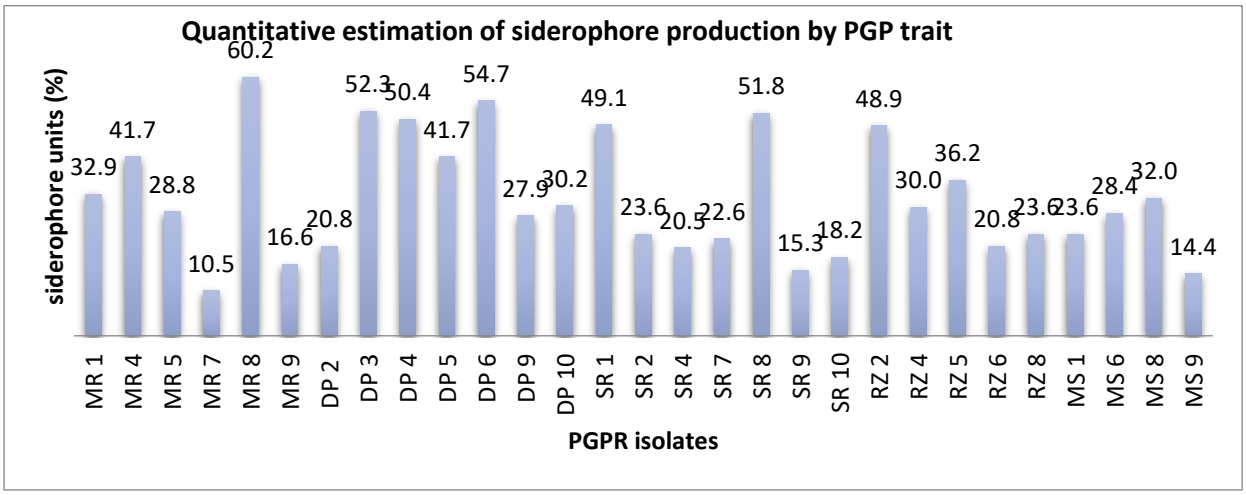


Fig.4. Quantitative estimation of siderophore production by PGP trait

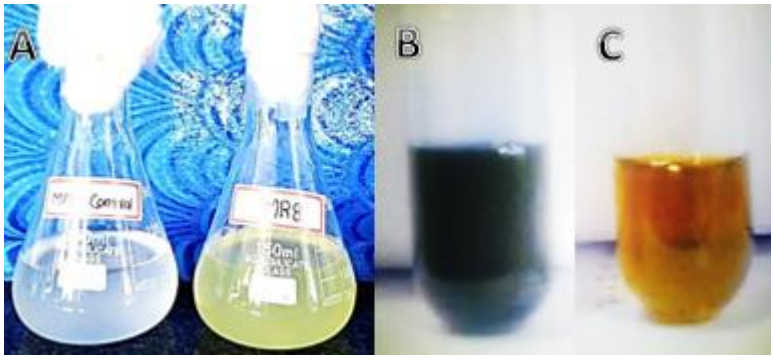


Fig.5. Detection of siderophore by CAS shuttle Assay, A- Invitro production of siderophore in MM9 medium, B- CAS assay solution C- Reduction of CAS assay solution by siderophore

Fig.6. PGPR strain MR8 Pseudomonas sp. isolated from rhizosphere soil. A- Streak plate culture of Pseudomonas sp. B-Streak plated *Pseudomonas sp.* Emitting fluorescence color under UV transilluminator.

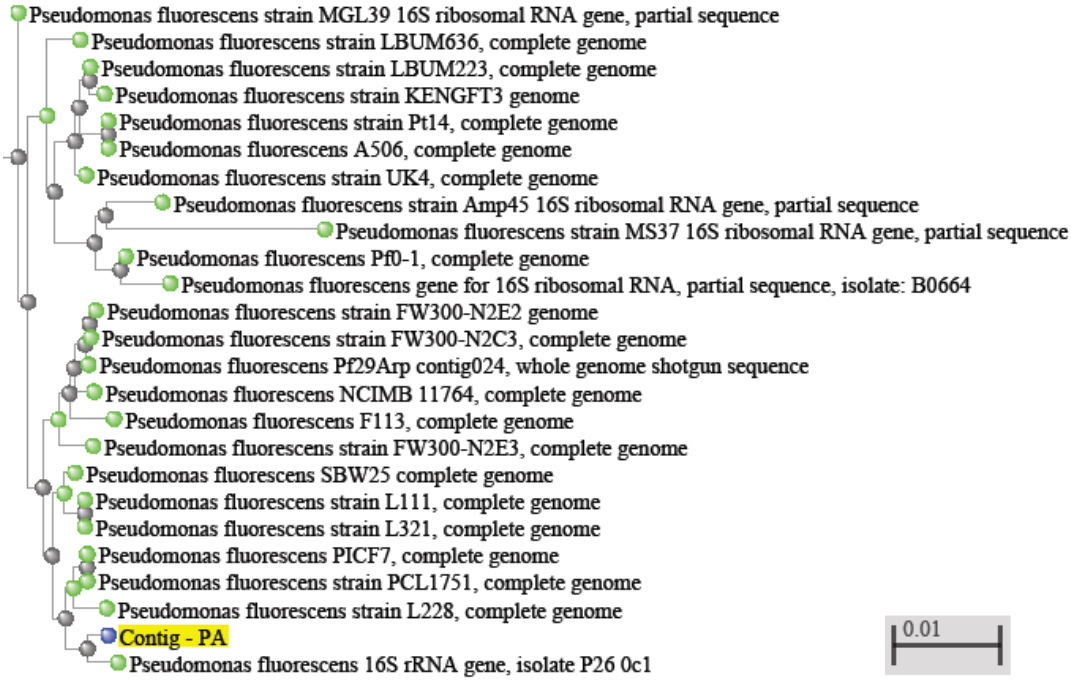


Fig.7. Neighbor-joining tree showing the phylogenetic relationship between 16S rDNA sequence of *Pseudomonas fluorescens* strain MR8 with other closely related bacteria.

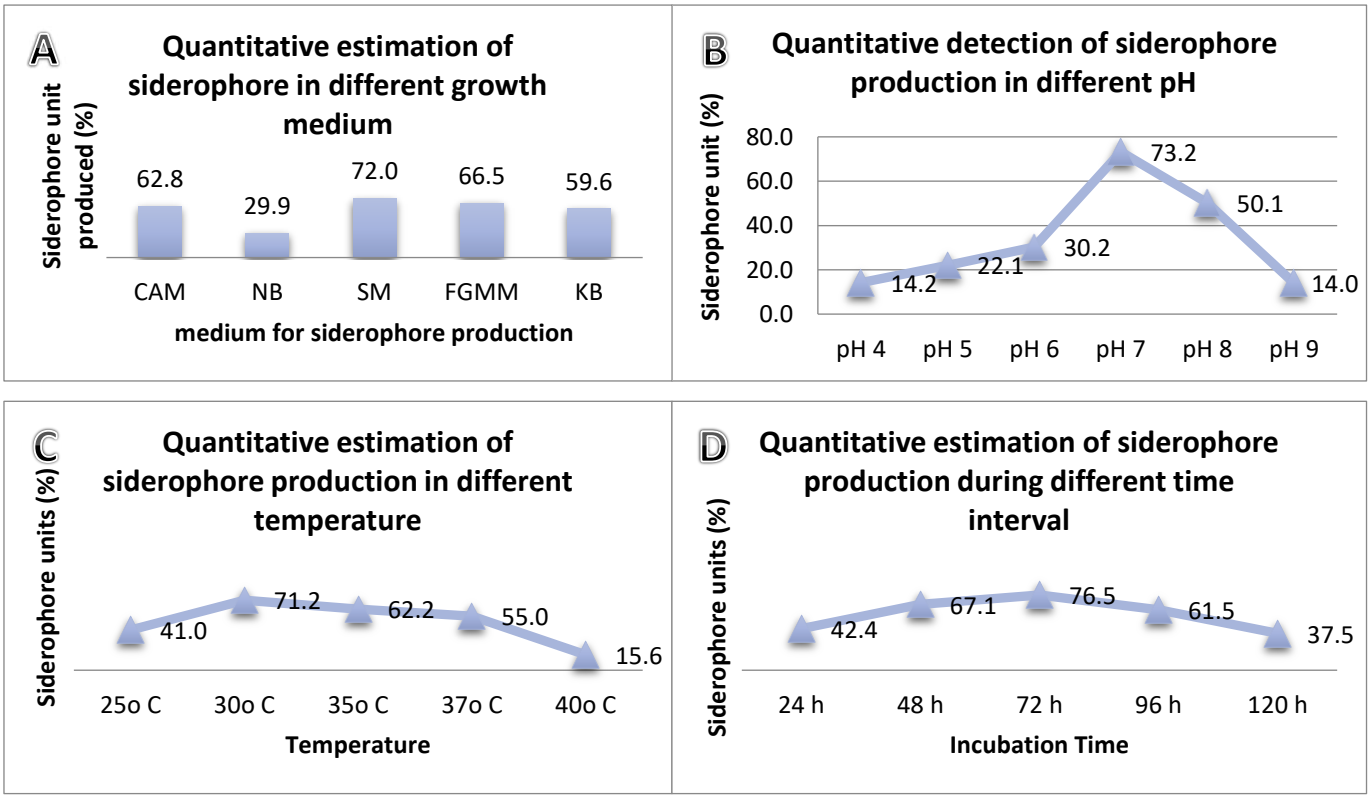


Fig.8. Quantitative estimation of siderophore production A- Different growth media, B- Different pH, C- Different temperature, D- Different time interval.

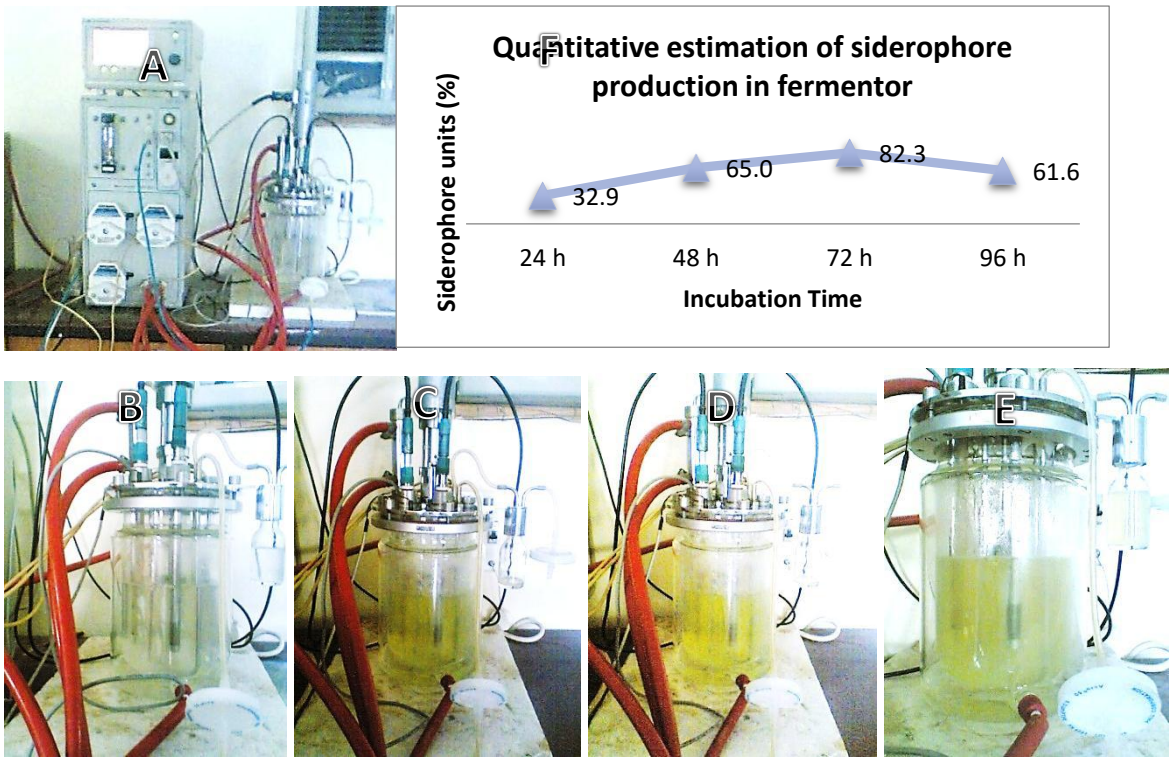
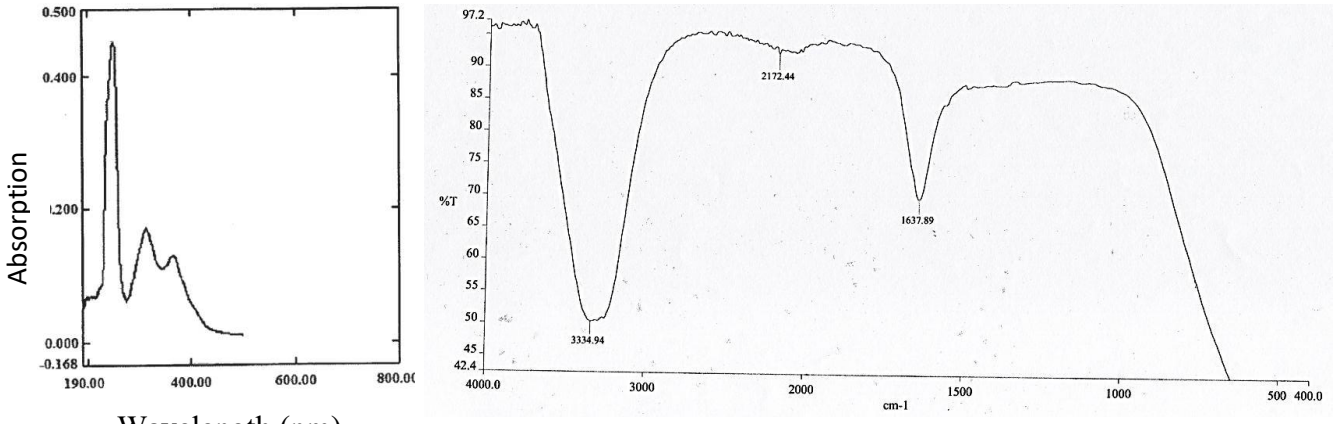


Fig.9. Fermentation with SM medium shows color changes during the production of siderophore (A- E) and F- quantitative estimation of siderophore.



A B

Fig.10. A. U V spectrum of siderophore B. FTIR spectrum of purified siderophore produced by *Pseudomonas fluorescens strain MR8*

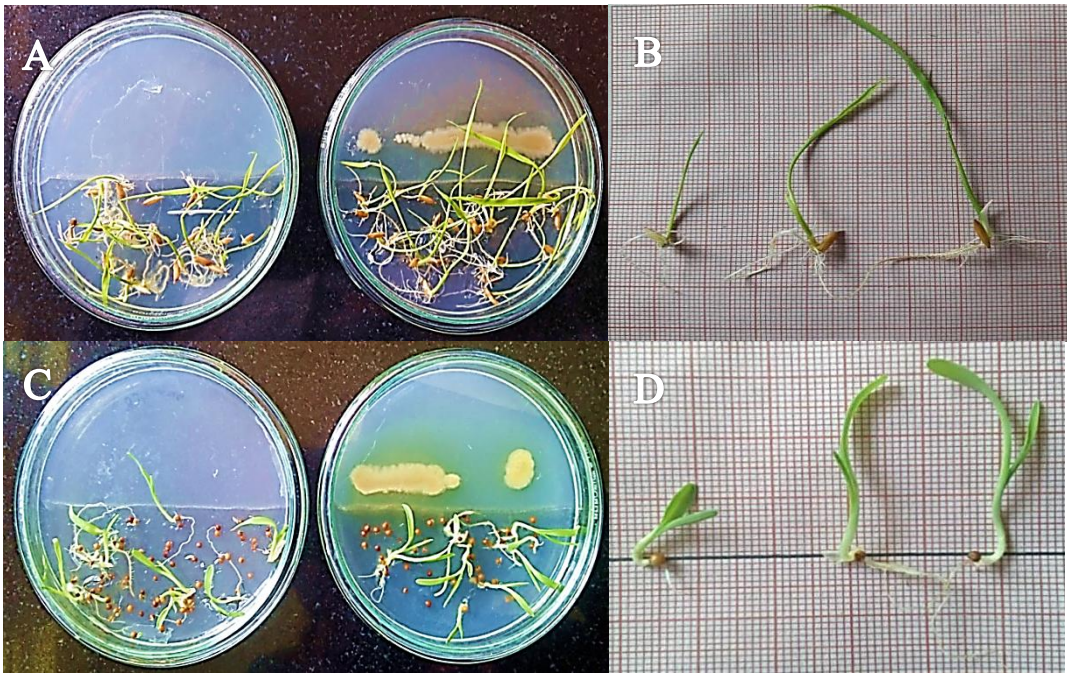


Fig.11. Invitro effect of *Pseudomonas fluorescens* MR8 after 10 days of inoculation with rice and finger millet. A- Increased germination, growth of Oryza sativa compared with control and treated with PGPR MR8. B- increased adventitious branches of rooting in PGPR treated (center) compared to control (left), C- significant increased growth of finger millet with PGPR treated and control and D- increased shoot and root length of finger millet (left- control, center- PGPR treated and right- siderophore treated.

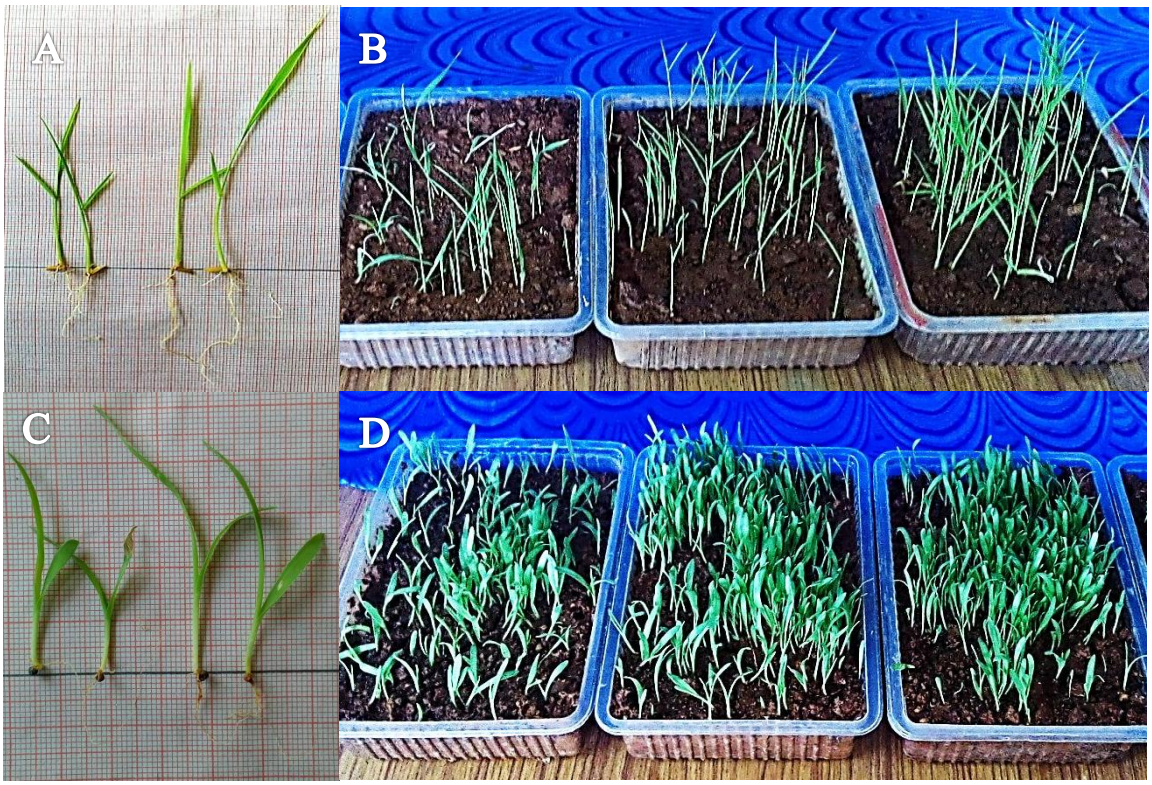


Fig.12. Effect of *Pseudomonas fluorescens* MR8 and siderophore after 10 days of inoculation with rice and finger millet in soil experiment. A- Increased germination, growth of Oryza sativa compared with control and treated with PGPR MR 8. B- increased growth of rice (left), PGPR treated (center) compared to control (left), C- finger millet seed germination in soil experiment, D- increased percent germination and growth of finger millet control (left), PGPR (center) and siderophore treated (right).

Supplementary Files

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- tables.docx