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RESEARCH ARTICLE

Isolation, identification and molecular characterization of Fluorescent Pseudomonads from the rhizosphere

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Abstract

Agrochemicals are widely used for plant disease control, but they leave toxic residues in agricultural commodities. Biological control plays an important role in sustainable disease management. Among biocontrol agents, Plant Growth Promoting Rhizobacteria (PGPR) are an important alternative, particularly Fluorescent Pseudomonads, which promote plant growth and aid in disease management. Bacterial antagonist belonging to *Pseudomonas* spp. are well-documented for their antagonistic abilities against various diseases across diverse pathosystems. Three *Pseudomonas* species are fluorescent, namely *P. aeruginosa*, *P. fluorescens* and *P. putida*. *P. aeruginosa,* the type species of the genus *Pseudomonas, exhibits greater uniformity due to fewer* biotypes compared to other fluorescent pseudomonads. Since *P. aeruginosa* is a human pathogen, *distinguishing it from P. fluorescens* is essential. The identification and diversity of fluorescent pseudomonad (FLP) isolates were investigated using the 16S rRNA gene sequence. PCR was performed to amplify the 16S rRNA geneusing primers 27F/1492R. Amplified DNA products were sequenced and analyzed using BLASTn (NCBI database) for species identification. Ten native FLP isolates were obtained. Nine of the ten isolates showed high bootstrap support value to *P. aeruginosa and and* one to *P. fluorescens*. Significant diversity was observed among isolates, as evidenced by a phylogenetic dendrogram constructed using CLUSTAL W.

Keywords: BLAST (BLASTn); CLUSTAL W; Fluorescent Pseudomonads; maximum residue limit; PCR

Introduction

India's population is steadily growing and is projected to reach 1.7 billion people by 2050 (1, 2). To meet this demand, a significant increase in food production is essential. According to the Food and Agriculture Organization (FAO), agricultural output must rise by at least 50 % under moderate economic growth scenarios (3-5). Therefore, enhancing yield per unit area and minimizing crop losses are critical priorities. In India, the climate, soil, topography and vegetation within the country vary significantly. Taking advantage of this, a variety of crop plants are cultivated across the country. The agricultural contribution is ~40 % of the nation's GDP and about 73 % of the population is engaged in it. Indian farming mainly depends on two monsoons (nearly 60 %), the southwest and northeast. The rainfall received in different parts of the country varies from less than 10 cm to over 700 cm and is distributed unevenly throughout the year, as is also the diversity in plant diseases (6).

Though the yield and productivity of the crops are enhanced after the green revolution, a certain range of crop losses occurs due to various diseases. Therefore, an increase in crop disease control is required to meet the global demand for the food supply (7, 8). Many plant diseases are known in India and each crop is infected by at least one pathogen and some are attacked by scores of them. A large quantity of fruits and vegetables are also infected by pathogens in the pre- and post-harvest stage in the field, as well as in transit and storage. There is therefore an urgent need to study these problems and develop a suitable technique to meet the challenge.

Fluorescent Pseudomonads are soil-borne, unicellular, rod-shaped, obligate aerobic bacteria known as pseudomonads, which are gram-negative, motile through one or more flagella and and do not produce spores, stalks, or sheaths. Fluorescent Pseudomonads (FLPs) belong to the genus *Pseudomonas*, family Pseudomonadaceae, order Gammaproteobacteria and phylum Proteobacteria. There are 191 species in this genus and among all pseudomonads are the most diverse and ecologically significant group of bacteria, which can survive harsh conditions, are ubiquitous and form associations with both plants and animals (9).

Among the *P. aeruginosa*, *P. fluorescens* and *P. putida* (10), *P. aeruginosa* is more uniform as it has less number of biotypes and it is also the type species of the genus *Pseudomonas* and as compared to other Pseudomonads fluorescent. There is zero availability of native fluorescent pseudomonad isolates that can be used against different major diseases, which will be adapted to local crops and climates. To fulfil this research gap, our investigation was performed. Given the urgent need for PGPR isolates adapted to local crops and climates, this study aims to molecularly characterize native fluorescent pseudomonad isolates.

Materials and Methods

Isolation of Fluorescent Pseudomonads

We collected the soil sample from a healthy plant rhizosphere in a diseased plot (above 60 % field was disease-infested in the case of the diseased plot and below 20 % in the case of the healthy plot). Soil samples were collected at a depth of 10 cm from different locations in the field. After shadow drying for 24 hr, 1 g of fine soil sample was taken, sieved and serially diluted up to 10^{-7}. At first, 1 g of soil was put into a test tube containing 10 mL of distilled water and mixed well. Then from that test tube, 1 mL of the mixture was taken and transferred to another test tube containing 9 mL of distilled water using a micro pipette. After continuing this process seven times, we got a dilution mixture of 10^{-7} and 100 µL of 10^{-5} and 10^{-6} dilution were put into the plates containing King's B media and spread.

The plates were incubated for around 1 to 2 days at 28 ± 2 °C. After 48 hr, the colonies were selected by fluorescence under UV light and those colonies were picked for making pure culture using a sterile loop and streaked on the plates containing King's B media. The plates were incubated for around 48 hr at 28 ± 2 °C and the pure culture plates were stored in a refrigerator for further use. The above procedure was followed for each soil sample.

Media for culturing Fluorescent Pseudomonads

King's B media was used for isolation, purification procedure and maintenance of various isolates of *Pseudomonas spp*. The composition of King's B media is mentioned in Table 1 (11). For the preparation of King's B Media, all the above chemicals were measured and added to 1000 mL of warm distilled water except glycerol and $MgSO_4$. After mixing all the ingredients pH was adjusted to 7.2 ± 0.2 and then $MgSO_4$ was added slowly to the mixture. The media was sterilized in an autoclave at 121.6 °C temperature with 15 psi pressure for 15 min. Before using the media for isolation of the *Pseudomonas* spp. we mixed glycerol after autoclaving with the cooled, molten media (500 °C is standard).

DNA (Deoxyribonucleic acid) extraction of Fluorescent Pseudomonad isolates

Bacterial isolates were used for DNA isolation. The genomic DNA from all chosen bioagent isolates was extracted using a DNA extraction kit (Nucleo-pore gDNA Fungal Bacterial Mini Kit (50) Cat. # NP-7006D). The amount, purity and concentration of the resulting DNA were verified using electrophoresis of agarose gels, followed by staining with ethydia bromide and visualization under ultraviolet light after extraction. PCR was performed using an Eppendorf Mastercycler Products were verified using electrophoresis of agarose gels and a ladder will be used as size markers. Then sequencing was done to confirm amplicon size (~1500 bp) and sequence identity (12). The detailed procedure is described below.

Table 1. Composition of King's B media

Proteose peptone	20 g
K2HPO4 (Dipotassium hydrogen phosphate)	1.5 g
MgSO4 (Magnesium sulphate)	1.5 g
Agar	15 g
Glycerol	10 mL
Distilled Water	1000 mL

Principle and Procedure

The Nucleo-pore gDNA Fungal/Bacterial Mini Kit was utilized for 15 min for the isolation of DNA from tough-to-lyse Gram (+) and (-) bacteria. Thrashing beads followed by mechanical lysis via bead-beating was used for homogenizing the sample without using organic denaturants or proteinases. Pure DNA was isolated using FB Spin column technology after two wash steps. The purified DNA is ideal for downstream molecular-based applications.

Detailed protocol

Cell lysis

Resuspend 50-100 mg (wet weight) of 48-hr-old bacterial cells in 200 µL sterile water in a thrashing bead tube. Add 750 µL lysis buffer FBL, vortex for 5 min and centrifuge (10000 × g, 1 min).

Column purification

Transfer 400 µL supernatant to an FB Shredder Column (8000 × g, 1 min). Mix filtrate with 1200 µL DNA Binding Buffer FBB, load 800 µL onto an FB Spin Column (10000 × g, 1 min) and repeat.

Wash/Elution: Wash with 200 µL Pre-Wash Buffer FBPW and 500 µL Wash Buffer FBW (10000 × g, 1 min each). Elute DNA in 100 µL Elution Buffer FEB (room temperature, 1 min) and store at -20 °C.

Gel electrophoresis

Gel electrophoresis system was carried out to confirm the presence of DNA.

Preparation of 50 X TAE buffer

At first, 24.2 g of Tris was dissolved in 50 mL of distilled water. 5.71 mL of glacial acetic acid was added to it and mixed well. Then 10 mL of 0.5 M EDTA was added in the solution and dissolved properly. Finally 100 mL volume was made by adding distilled water.

Preparation of 1 X TAE buffer

8 mL of 50X TAE buffer was diluted in 392 mL of distilled water to prepare 400 mL of 1X working solution.

Preparation of agarose gel

Agarose gel (1 %) was prepared by dissolving 0.5 g of agarose in 50 mL of 1 X TAE Buffer in a flask. The mixture was boiled in a microwave oven (a power level of 900-1200W for a few minutes) until it became completely transparent (need handling, monitoring, ventilation and container type precautions).

Casting agarose gel

After cooling the mixture to about 50-55 °C, 2.5 µL ethidium bromide (ethidium bromide is hazardous; handling precautions are needed) was added to the solution and mixed slightly. The agarose mixture (transparent) was poured on a casting tray on which a well-forming comb had been placed at a 2 mm height. It was left for 20-30 min for polymerization.

Setup of electrophoresis

After polymerization of the gel, the combs were removed to create wells and then it was transferred in a tank of gel electrophoresis containing appropriate amount of 1 X TAE Buffer (enough to submerge the gel). 2 µL of extracted DNA from each sample was loaded with 2 µL of DNA loading dye (6X TriTrack) in the wells of the gel and allowed to run at 50 V for 45 min. After that, the gel was observed on the UV Transilluminator to see the presence or absence of DNA in the samples.

PCR amplification

Detection of the 16S rRNA gene was done by PCR amplification, which was set up using 16S rRNA gene-specific primers - 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) as forward and reverse primers, respectively. A total of 25 µL reaction mixture was prepared for each sample. The composition of the PCR reaction mixture is as Table 2. After adding 2 µL of DNA in each tube, reaction mixture was mixed well by mini-centrifuge and all the tube containing the reaction mixture were placed in a thermal cycler which was set at the amplification environments (Initial denaturation - 94 °C with 3 min, denaturation - 94 °C with 30 sec, annealing - 50 °C with 45 sec, extension - 72 °C with 1 min 50 sec and final extension - 72 °C with 10 min).

After PCR amplification gel electrophoresis was done as described before. 2 µL of PCR amplified product of each sample was loaded with 2 µL of DNA loading dye (6X TriTrack) in the wells of the gel keeping the first well reserved for ladder where we put 2 µL of DNA ladder (GeneRuler 1 kb Plus) to check the presence of specific gene and amplified product size. Then we sent the PCR products of each sample for sequencing to check for phylogenetic analysis.

Characterization and sequence analysis of Fluorescent *Pseudomonad* isolates

The area of the amplified 16S rRNA gene, which is a specific region of Pseudomonas of the Fluorescent Pseudomonads isolates, was amplified with the same primer pair in the same procedure as mentioned above and sequenced directly by the Sanger sequencing technique at Barcode Biosciences, Bengaluru, Karnataka. Then the sequences were compared to those extracted from GenBank using the BLASTN programme in NCBI (National Centre for Biotechnology Information) website (e.g., "≥ 97 % identity for species-level identification") and aligned using the CLUSTAL W programme using MEGA 11 software (Settings for CLUSTAL W Alignment - gap opening penalty is 15.00 and gap extension penalty is 6.66. These values are the same in pairwise as well as multiple alignment. Settings for phylogeny reconstruction - statistical method: neighbour joining, mode: maximum composite likelihood, substitutions to include: transition + transversions, substitution type: nucleotide, rates among sites: uniform, pattern among lineages: homogeneous, gaps treatment: pairwise deletion, number of threads: 3).

Table 2. Composition of PCR mixture

Master mix	12.50 (Amount in µL)
Forward primer (27F)	1.00 (Amount in µL)
Reverse primer (1492R)	1.00 (Amount in µL)
Nuclease free water	8.50 (Amount in µL)
Template DNA	2 (Amount in µL) of respective DNA in each tube
Total	25 (Amount in µL)

Computation of diversity indices for Fluorescent *Pseudomonad* isolates

The diversity indices for 10 Fluorescent *Pseudomonads* isolates, like - Shannon Index and Simpson Index, were calculated in R software using phyloseq and vegan packages and by using the following formula.

$$\text{Shannon Index (H)} = -\sum_{i=1}^{s} p_i \ln p_i \qquad \text{(Eqn. 1)}$$

$$\text{Simpson Index (D)} = 1/\sum_{i=1}^{s} p_{i\wedge 2} \qquad \text{(Eqn. 2)}$$

(Where, S: Total number of species, p_i: Proportion of the i^{th} species in the community (i.e., $p_i = n_i/N$), n_i: Number of individuals (or sequences) of species *i,* N: Total number of individuals (or sequences) across all species, ln: Natural logarithm) (23, 24).

Results

Molecular characterization of FLP isolates

Genomic DNA was successfully isolated from 10 FLP isolates and confirmed via 1 % agarose gel electrophoresis (Fig. 1). The 16S rDNA region was amplified using primers 27F AGAGTTTGATCMTGGCTCAG)/1492R), yielding ~1500 bp products. Sanger sequencing of these amplicons revealed genetic diversity among the isolates, as evidenced by phylogenetic analysis.

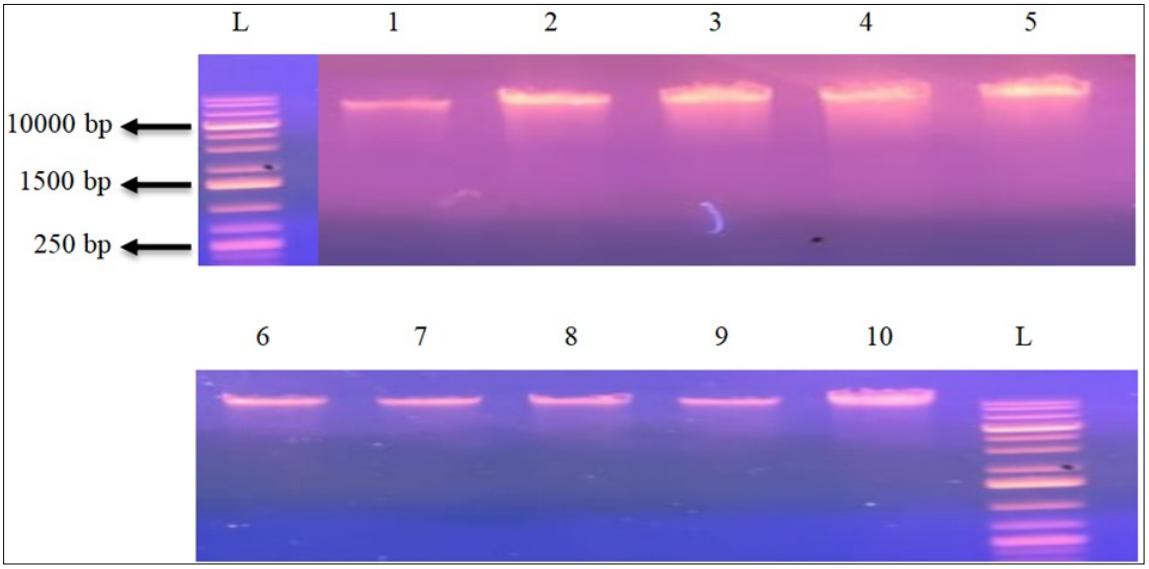

Fig. 1. Genomic DNA of different FLPs isolates (L-Ladder,Details of 1-10 in Table 1).

Genomic DNA isolation of FLP isolates

All 10 FLP isolates yielded high-molecular-weight genomic DNA, as visualized by clear bands on 1 % agarose gels (Fig. 1).

Polymerase chain reaction analysis

PCR amplification with universal 16S rRNA primers (27F/1492R) produced ~1500 bp bands, confirming target gene amplification (Fig. 2). Sequencing and phylogenetic analysis of these amplicons revealed isolate-level diversity.

Design and analysis of phylogenetic structures derived from nucleotide sequences of the 16S rRNA

The 16S rRNA sequences from 10 FLP isolates were compared to NCBI GenBank entries using BLASTn (≥ 97 % identity threshold). A Neighbour-Joining phylogenetic tree (bootstrap = 1000) was constructed to assess evolutionary relationships (Fig. 3). Here, the recommendation to use 1000 bootstrap replicates in phylogenetic analysis is based on achieving statistical reliability and stability in the support values for branches in the tree. Bootstrap values represent the confidence or support level for each branch in a phylogenetic tree. More replicates (like 1000) provide more accurate estimates of the true confidence intervals for each clade. Fewer replicates (e.g. 100 or 250) may produce unstable or inflated values, especially for closely related taxa.

The phylogenetic tree grouped isolates into two primary clusters (82 % bootstrap support value). Because there's high genetic divergence from the reference isolates (e.g., in 16S rRNA, housekeeping genes), they will form a separate clade or branch. Isolates from unique local environments (e.g., specific soil, rhizosphere, climate) often show ecological adaptation. This can result in distinct evolutionary trajectories and thus separation from commonly sequenced reference isolates. One comprised nine FLP isolates closely related to *P. aeruginosa* (including FLP 2020-1/2020-2, morphologically identified as *P. fluorescens*, but after molecular identification, FLP 2020-1 was only proved to be *P. fluorescens*) and another contained reference isolates (Fig. 3; Table 3). Bootstrap support value percentages between subclusters ranged from 60 % (Brinjal-4/Wheat rhizosphere isolates) to 81 % (Pant-Brinjal/Barley group). Notably, Turmeric/Mustard and Pant-Brinjal/Barley isolates formed tightly related pairs. After performing diversity indices for 10 isolates, we found a Shannon index of 0.639 and a Simpson index of 0.5.

Discussion

Molecular characterization of Fluorescent *Pseudomonads* isolates

Molecular characterization is an important process that provides valuable data to detect variation at DNA level and to assess genetic diversity among the various isolates. Previous works have focused on the molecular identification of *Pseudomonas* isolates (14), which can fix nitrogen, by rrs (16S ribosomal RNA gene) sequence analysis with specific primers - FGPS4-281 bis (AGA GTT TGATCC TGG CTC AG) and FGPS1509'-153 (AAG GAG GTG ATC CAG CCG CA) and reported similarity with an unidentified marine bacterium (99 %), uncultured bacteria (98 %), *Pseudomonas spp.* PCP2 (98 %) and *Pseudomonas alcaligenes* (97 %).

Previous studies highlight the utility of 16S rRNA sequencing for *Pseudomonas* characterization. For instance, studies identified phosphate-solubilizing *P. mendocina*, *P. stutzeri* and *P. putida* from saline soils using RAPD and 16S rRNA analysis (15). Similarly, previous findings amplified ~1500 bp fragments with universal primers (27F/1492R), identifying P. *aeruginosa* and other pseudomonads (16-18). These findings align with our observations of 16S rRNA amplicons (~1500 bp) from FLP isolates (Fig. 2). Research indicates that molecular phylogenetic analysis and evolutionary relationship analysis of *Pseudomonas* isolates by 16S rRNA primer pair and comparing the sequences of the isolated *Pseudomonas* isolates with reference isolate sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank public database (18, 19). Notably, research indicates that Pseudomonas-specific primers (16SF/16SR) for isolate verification, underscoring the versatility of PCR-based methods in Pseudomonas research (20). Research observed the genetic diversity and phylogenetic analysis among multidrug-resistant *Pseudomonas* spp (21). Their work revealed distinct clusters that highlight significant geographic linkages and genetic variability among the isolates. Research characterised and identified *Pseudomonas spp.* AW4, an arsenic-resistant and plant growth-promoting bacterium isolated from the soybean (*Glycine max* L.) rhizosphere and found that a *Pseudomonas* sp. AW4 formed a monophyletic clade with *P. urmiensis* SWRI10, presenting 3.08 % of unique genes against this reference isolate. More than 70 % of AW4 genes were also shared with *P. oryziphila* isolate 1257 NZ and with *P. reidholzensis* isolate CCOS 865 (22).

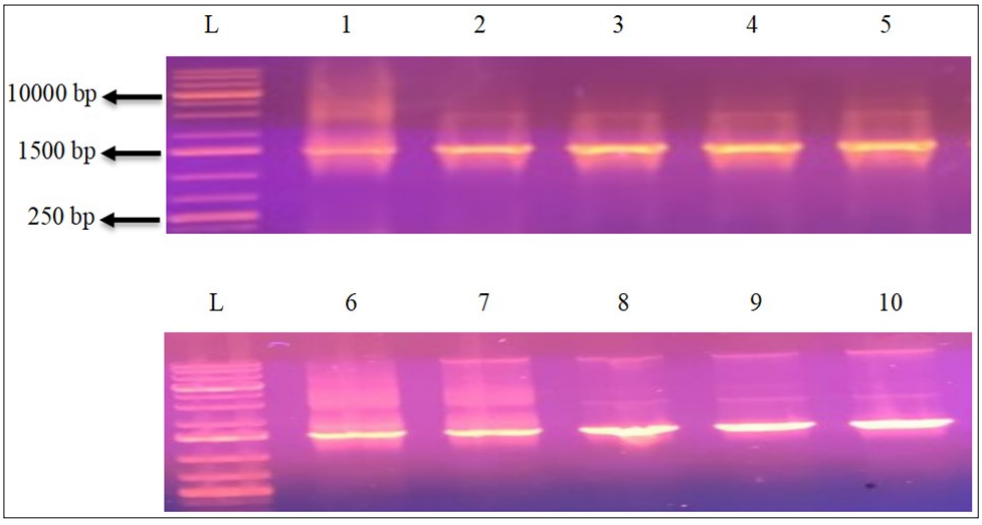

Fig. 2. Amplification of gene encoding 16S rRNA (L - Ladder, Details of 1-10 in Table 1).

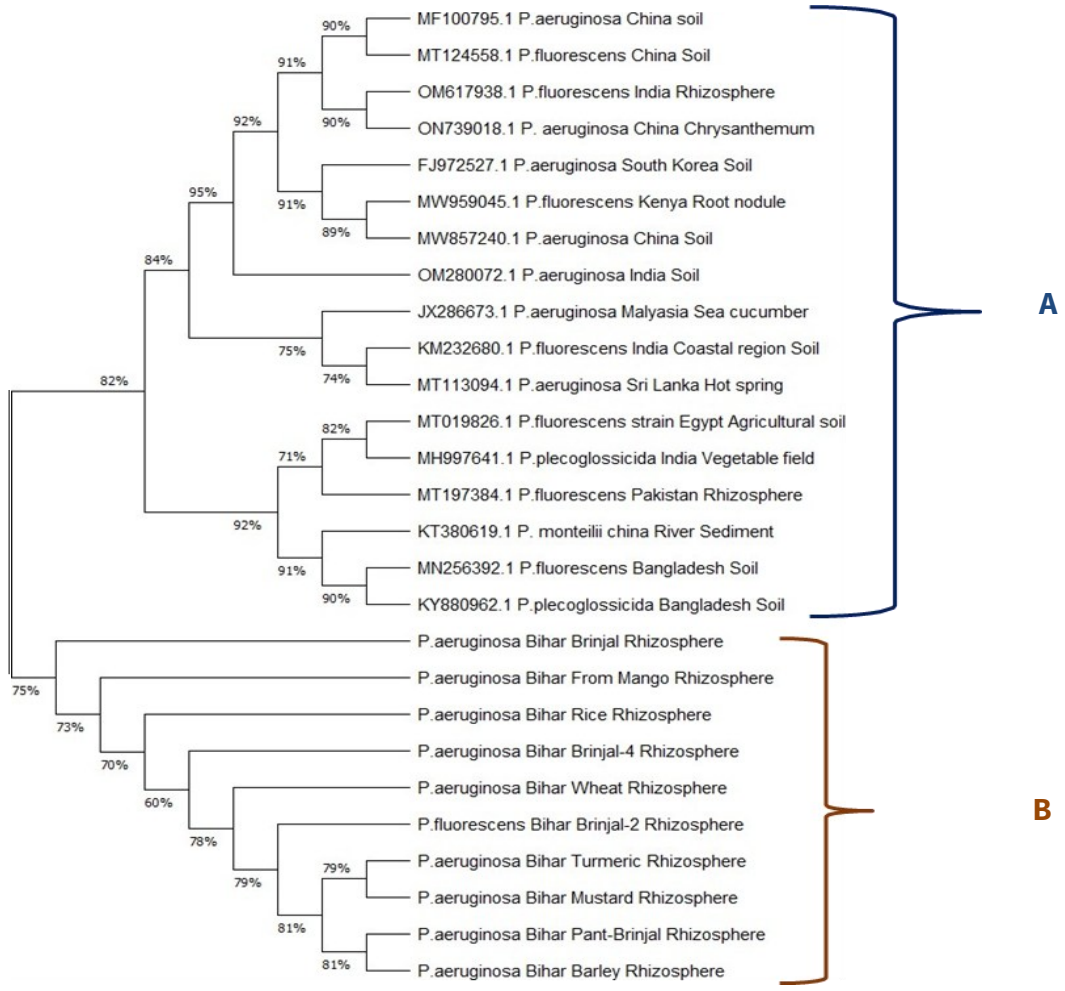

Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. This analysis involved 27 nucleotide sequences. Evolutionary analyses were conducted in MEGA11(Here **A** represents Clade I and **B** represents Clade II) (13).

Table 3. Fluorescent Pseudomonads isolates, used in phylogenetic analysis

Sl. No.	Fluorescent Pseudomonads isolates	Origin	Code	Blast percentage identity	Sequenced product length	Accession number
1.	*Pseudomonas spp.*	Brinjal rhizosphere	PF	*Pseudomonas aeruginosa* (99.86 %)	1215 bp	PV820014.1
2.	*Pseudomonas spp.*	Brinjal rhizosphere	Br2 (FLP 2020-1)	*Pseudomonas fluorescence*(99 %)	1239 bp	PV820015.1
3.	*Pseudomonas spp.*	Brinjal rhizosphere	Br4 (FLP2020-2)	*Pseudomonas aeruginosa* (98.9 %)	946 bp	PV820016.1
4.	*Pseudomonas spp.*	Brinjal rhizosphere	Pt	*Pseudomonas aeruginosa* (98.99 %)	1274 bp	PV820017.1
5.	*Pseudomonas spp.*	Rice rhizosphere	Ri	*Pseudomonas aeruginosa* (98.7 %)	1101 bp	PV820018.1
6.	*Pseudomonas spp.*	Wheat rhizosphere	Wh	*Pseudomonas aeruginosa* (98.8 %)	1224 bp	PV820019.1
7.	*Pseudomonas spp.*	Barley rhizosphere	B	*Pseudomonas aeruginosa* (98.8 %)	1276 bp	PV820020.1
8.	*Pseudomonas spp.*	Mustard rhizosphere	Mu2	*Pseudomonas aeruginosa* (98.7 %)	1285 bp	PV820021.1
9.	*Pseudomonas spp.*	Mango rhizosphere	Ma	*Pseudomonas aeruginosa* (99.13 %)	1154 bp	PV820022.1
10.	*Pseudomonas spp.*	Turmeric rhizosphere	Tu	*Pseudomonas aeruginosa* (98.87 %)	1239 bp	PV820023.1

In this table codes are used only for giving the isolates a short name

In this study, all 10 FLP isolates yielded ~1500 bp 16S rRNA amplicons (Fig. 2), confirming their identity as rhizosphere-associated *Pseudomonas* spp. Research indicates similar amplicon sizes for *P. putida* and *P. fluorescens*. The consistency in band size across studies validates the specificity of 27F/1492R primers for Pseudomonas detection. We found most of the isolates as *P. aeruginosa* over *P. fluorescens* because *P. aeruginosa* can be a dominant species in our local environment, as fields are with organic-rich matter and our locality has a moist environment, which is helpful for the dominance of *P. aeruginosa.* Phylogenetic analysis revealed two major clusters (82 % bootstrap support): one comprising nine FLP isolates closely related to *P. aeruginosa* (including FLP 2020-1/2020-2, morphologically identified as *P. fluorescens*) and another containing reference isolates from diverse geographical regions (Fig. 3). This clustering suggests potential niche adaptation, as seen in *P. aeruginosa* isolates from soil (OM280072.1) and marine environments (JX286673.1). The high similarity among FLP isolates may reflect local environmental selection pressures, consistent with previous research, which indicates geographical influences on Pseudomonas evolution.

After performing diversity indices, also Shannon diversity index (H) of 0.693 indicates moderate to low diversity in the community and it also says that one species is dominant and another is rare. A Simpson diversity index (D) of 0.5 also says that one species is dominant and another is rare. These results of our investigation are also supported by the previous research observation (23, 24).

Conclusion

The creation of biological control agents has drawn attention as an alternate, eco-friendly method of defending horticultural and crop plants against phytopathogens due to consumer and environmental concerns. One such biological control agent that has been shown to work is *Pseudomonas fluorescens*. The present experiment was carried out to characterize the native Fluorescent Pseudomonads isolates collected from the agroclimatic zone of Bihar. Nine of the ten FLP isolates that were evaluated showed the most resemblance to *Pseudomonas aeruginosa*, while FLP 2020-1 was determined to be *Pseudomonas fluorescens* (Which is our novelty of research work, because we successfully provided the native isolate of *Pseudomonas fluorescens* through our research, which was not documented previously). *P. fluorescens* is a non-pathogenic, naturally occurring soil bacterium. *P. aeruginosa*, on the other hand, is an opportunistic human pathogen known to cause infections in immunocompromised individuals. Due to biosafety concerns, *P. aeruginosa* is not recommended for use in open agricultural environments or biofertilizer formulations. *P. fluorescens* can be used as not only a bio-control measure of diseases but also as plant growth-promoting rhizobacteria for achieving sustainable agriculture and our goal should be to make them into reliable, assessable product for farmers at commercial level.

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Authors' contributions

RS carried out the field experiment, analysed data and prepared this manuscript. JNS carried out molecular analysis. SA carried out a statistical analysis. MP mentored research programme. AK carried out data interpretation. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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