Morphological, Biochemical and Molecular Characterization of Plant Growth Promoting Rhizobacteria with Synergistic Effect Against Fusarium oxysporum

ABSTRACT

Currently, plant growth-promoting rhizosphere bacteria (PGPR) are heavily exploited as microbial inoculants in agricultural production among which Pseudomonas sp. and Bacillus sp. are widely used in plant growth promotion and disease control as excellent inoculum strains. Plant growth-promoting rhizobacteria has the potential to be used in agriculture to promote plant growth and health through various mechanisms. Therefore, identifying novel strains tailored to specific agricultural requirements continues to be a key area of research. In the present research work, Pseudomonas fluorescens, Pseudomonas putida, and Bacillus subtilis isolates from soybean rhizosphere have been chosen for further research based on their morphological, biochemical, and molecular characterization. All three isolates have been identified as per Bergey's Manual for the determination of bacteriology. All isolates tested positive for starch hydrolysis, citrate utilization, catalase activity, indole production, gelatine hydrolysis, and tetrazolium reduction. Additionally, the 16S rRNA gene sequences of the various potential isolates were validated as P. fluorescens VNMKV1, P. putida VNMKV1, and B. subtilis VNMKV1. The potential of these novel strains of plant growth-promoting rhizobacteria as biocontrol agents was explored by screening against soil-borne pathogenic fungi highlighting their potential to be used as bioinoculant agents.

Keywords: [PGPR, 16S-rDNA, antagonistic, DNA sequencing, GenBank]

1. INTRODUCTION

The intensification of agricultural production due to the rising global population has led producers to increasingly depend on agrochemicals for crop protection and economic stability. However, the escalated use of these chemicals has resulted in numerous adverse effects. Furthermore, the increasing costs of pesticides and the growing consumer demand for pesticide-free food have spurred the search for alternative solutions[1]. Consequently, biological control is currently explored as an option or supplement to minimize chemical usage in agriculture [2]. One promising approach to enhancing plant yields without environmental harm involves the use of specific microorganisms. These beneficial microorganisms can safeguard plants from pathogenic microorganisms prevalent in agricultural soils [3]. The soil microbiome constitutes the greatest source of biodiversity on the planet Earth [4]. Soil bacteria play crucial roles in nutrient cycling and have been employed in agricultural practices for many years [5].

Free-living soil microbes known as plant growth-promoting rhizobacteria (PGPR) promote plant root development and growth by employing various direct and indirect mechanisms [6,7].The direct mechanisms of PGPR involve phosphate solubilization, nitrogen fixation, siderophore production, and regulation of plant growth hormones, such as auxins, gibberellins, cytokinin, and ethylene. This is achieved through the production of the PGPR-specific enzyme ACC deaminase, which enhances the uptake of phosphorus, nitrogen, and iron, ultimately promoting plant growth [8]. Several indirect mechanisms, including induced systemic resistance (ISR), antibiosis, nutrient competition, parasitism, and the production of metabolites such as hydrogen cyanide and siderophores that inhibit harmful rhizobacteria, also contribute to promoting plant growth. Additionally, it enhances the rate of germination, growth of shoot and root, total plant biomass, fodder and fruit yield, which can serve as sources of biofertilizers [9].

A putative PGPR is considered a PGPR when it could positively affect the plant after inoculation, exhibiting robust competitive abilities against native rhizosphere microbial communities. Generally, 2-5% of bacteria in the rhizosphere consist of PGPR [10]. Plant Growth-Promoting Rhizobacteria (PGPR) are pivotal for environmentally sustainable farming, Marking an optimistic trend for the future. These bacterial species adhere to soil particles through simple ion exchange, enhancing soil fertility by emitting inorganic nutrients from organic reserves at a level optimal for accelerated plant growth. Prominent genera of these bacteria, such as *Acetobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Derxia*, *Enterobacter*, *Gluconacetobacter*, *Klebsiella*, *Herbaspirillum*, *Ochrobactrum*, *Pantoea*, *Pseudomonas*,

Rhodococcus, *Serratia*, *Stenotrophomonas*, and *Zoogloea*, have been the focus of extensive research over the years [1]. *Pseudomonas* sp. and *P. putida* are notable for their roles in plant health, pathogen suppression and phosphate solubilization [12,13]. *Bacillus subtilis*, a Gram-positive bacterium, benefits plants through phytohormone production, pathogen suppression, and nutrient uptake [14,15].

To identify pure bacterial cultures, the process starts with examining their morphology and cultural traits, such as gram staining and colony characteristics. Initial classification relies on biochemical tests to evaluate the metabolic activities of the bacteria, which is essential for determining their identity. Advanced classification methods involve molecular techniques. This process includes isolating genomic DNA, amplifying specific DNA sequences through polymerase chain reaction (PCR), and sequencing the 16S ribosomal RNA (rRNA) gene. The 16S rDNA gene sequencing method is favoured because it is present in nearly all bacteria, remains stable throughout evolutionary history and provides a sequence length of ~1,500 base pairs which is sufficient for analysis [16]. Sequences are compared against databases like GenBank using tools such as BLAST, CLUSTAL XI and phylogenetic trees or dendrograms are constructed to establish the genus and identify the closest relatives of the strain [17,18].

Advancements in sequencing and DNA fingerprinting technologies have significantly improved the detection and identification of target microbes, reducing reliance on extensive morpho-physiological tests [19]. PCR-based techniques complement traditional methods for identifying and quantifying microorganisms, aiding efforts to enhance food safety and environmental quality. The application of plant growth-promoting rhizobacteria (PGPR) is crucial for reducing chemical inputs in agriculture. PGPR, including phosphate-solubilizing bacteria such as *P. fluorescens*, *P. putida* and *B. subtilis* have been shown to enhance plant growth, seed emergence and crop yield. This study aims to characterize these novel PGPR strains morphologically, biochemically and molecularly using 16S rRNA gene sequences to better understand their roles in promoting plant growth and enhancing nutrient availability.

2. MATERIALS AND METHODS

2.1 Collection of Microbial Cultures

Microbial cultures of plant growth-promoting rhizobacteria *viz*., *Pseudomonas fluorescens* VNMKV1*, Pseudomonas putida* VNMKV1, *Bacillus subtilis* VNMKV1 were collected from the 'Biomix' Research and Production Unit of Department of Plant Pathology, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani (India).

2.2 Subculturing of Microbial Cultures

Bacterial subculturing from pure cultures was performed under aseptic conditions within a laminar airflow chamber. An inoculating loop was sterilized by heating it in a flame, then used to carefully transfer the bacterial culture onto nutrient agar plates using a streaking technique. The petri plates were subsequently sealed with parafilm. The wire loop was reheated and a single bacterial colony was then transferred to a falcon tube containing a nutrient broth medium. Both the inoculated plates and falcon tubes were incubated at 37°C. After 24 hours, bacterial colonies developed on the agar plates and turbidity in the nutrient broth indicated successful bacterial growth.

2.3 Morphological Characterization of PGPR

Morphological characterization of rhizobacterial isolates was conducted as described by Swarupa *et al*. in their study[20]. The bacterial strains were incubated on nutrient agar at 37°C for a period of 24 hours and then subjected to microscopic examination *viz*., colony morphology, including size (<1 mm, 1-2 mm, 2-3 mm, or >3 mm), shape, color, consistency (mucous, fluid, or mycelial), surface aspect (smooth or rough), brightness (bright, translucent, or opaque) and growth speed. The bacterial growth rate was observed as very fast (> 24 hours), fast (24-48 hours), intermediate (24-48 hours), slow (36-96 hours), or very slow (> 96 hours). The gram reaction was performed following standard protocol [21].

2.4 Biochemical Characterization of PGPR

Biochemical characterization of the bacteria was carried out for the parameters *viz*., starch hydrolysis test, gelatine hydrolysis test, catalase test,tetrazolium test,indole production test, citrate utilization test according to Bergey's Manual of Systematic Bacteriology [22].

2.4.1 Starch hydrolysis test

Starch agar was prepared, autoclaved, and poured into petri plates. Bacterial isolates were inoculated onto the starch agar and were incubated at 37°C for a period of 24 hours. Following incubation, the plates were filled with Gram's iodine solution. The presence of clear zones surrounding the bacterial colonies indicated positive starch hydrolysis [23].

2.4.2 Gelatine hydrolysis

Nutrient gelatine was prepared, autoclaved, and dispensed into test tubes. The test organisms were inoculated into the nutrient gelatine and incubated at 45°C for 48 hours. Gelatine hydrolysis was assessed by chilling the tubes in ice or a freezer for 30 minutes. A positive result was indicated by the absence of solidification in the gelatine [24].

2.4.3 Catalase test

This assay was performed to identify the catalase activity in bacterial colonies. Fresh cultures of pure isolates were placed on glass slides followed by the addition of one drop of

30% hydrogen peroxide (H_2O_2). The immediate evolution of gas bubbles signified a positive result for catalase activity [25].

2.4.4 Tetrazolium test

The tetrazolium test detects hydroxamic acid by its ability to reduce tetrazolium salt in the presence of strong alkali. In a 100 µl bacterial sample, a pinch of tetrazolium salt was added with 1-2 drops of 2N NaOH. The rapid development of a deep red colouration demonstrated the presence of hydroxamate siderophores [26].

2.4.5 Citrate utilization test

The PGPRisolates were inoculated on Simmon's citrate agar media and placed for incubation at 28±2℃ for 24 hours. A positive reaction in this test indicated by the change in color from green to blue [27].

2.4.6 Indole production test

The sterilized nutrient broth was inoculated with PGPR isolates and incubated at 28 ± 2°C for 48 hours. After incubation, 10 drops of Kovac's reagent was added to each tube. Isolates that exhibited a red color ring were noted as positive for indole production [28].

2.4.7 Antagonistic Activity

All PGPR were studied for their antagonistic ability against the soil-borne plant pathogen *Fusarium oxysporum* by following the dual culture technique [29].Initially, the bacterial isolates were inoculated onto the nutrient agar media plates and incubated at 37°C for 24 hr. A loopful of each bacterial isolate was inoculated onto the potato dextrose agar around the edges of plates, which had been pre-inoculated with a 5 mm mycelial disc of the 5 days old test pathogen. A control plate was prepared by only placing the inoculum of the mycelia of a pathogen in the center, without inoculation of plant growth-promoting rhizobacteria. The suppression of mycelial growth of the test pathogens was monitored following incubation at 28°C for 5 days.

The percentage of growth inhibition compared to the control was determined using the following formula:

$$\text{Percentage inhibition (\%)} = \frac{\text{Pathogen growth in control (mm) - Pathogen growth in treatment (mm)} \times 100}{\text{Pathogen growth in control (mm)}}$$

Note: In this case, the percent inhibition in the control was considered as zero.

2.5 Molecular Characterization of PGPR

2.5.1 Isolation of bacterial genomic DNA

The genomic DNA of PGPR strains was isolated using a standard procedure given by Nour *et al*. in their study [30]. Overnight-grown 6 ml of bacterial culture were centrifuged at 10,000

rpm for 2 minutes in a 1.5 ml Eppendorf tube. The pellet was resuspended in 600 µl of TE buffer, this was followed by the addition of 3 µl proteinase K of (20 mg/ml) and incubated for 1 hr at 37°C. Subsequently, 0.1 ml of NaCl (5M) was added, which was followed by the addition of 80 µl solution of CTAB/NaCl. The incubation of the mixture was carried at 65°C for 10 minutes followed by treatment of an equal volume of phenol: chloroform: isoamyl alcohol and the mixture was subjected to centrifugation at 12,000 rpm for 7 minutes. The upper phase was transferred to a fresh tube and the extraction with chloroform: isoamyl alcohol was repeated twice. The precipitation of DNA was done by adding 1.5 volumes of isopropanol to the fresh tube with upper aqueous phase followed by 30 minutes of incubation at room temp and centrifuging at 12,000 rpm for 10 minutes. Thus, the formed pellet of DNA was washed with 70% ethanol, dried inside a laminar airflow cabinet and suspended in 0.1 ml of $T_{10}E_1$ buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) followed by storing the DNA at -20°C. The purity of DNA was checked by agarose gel electrophoresis and used as template DNA in PCR amplification of the 16S rDNA gene.

2.5.2 16S rDNA gene amplification of PGPR isolates

The 16S rDNA gene is found in all bacterial species and archaea, rendering it a universal marker for the identification and classification of prokaryotic organisms. The 16S rRNA gene contains variable regions that differ among species and highly conserved regions that are similar across the different species. The variable regions provide the necessary sequence diversity to differentiate between closely related species whereas conserved regions allow for the design of universal primers for 16S rDNA amplification. Therefore, the detection of bacteria was conducted using 16S rDNA primer (Table 1). The primers were synthesized at Eurofins Genomics Private Limited Bengaluru, India). The 16S rRNA gene was amplified on a PCR machine, with a total reaction volume of 25 µl. The PCR mixture contained 2.5µl of (10x Taq Buffer), 1.5 µl of (25 mM $MgCl_2$), 0.3 µl (10 mM) dNTPs, 1µl of (10 pM) forward primer, 1µL of (10 pM) reverse primer, 0.33 µl of (1U/µl) *Thermus aquaticus* DNA polymerase, 2µl template DNA, and 16.37µl of nuclease-free water.

Table 1: List of primers used for identification of PGPR isolates

Primer	Sequence 5’ to 3’	Tm (°C)
Y1	TGGCTCAGAACGAACGCTGGCGGC	69
Y3	TACCTTGTTACGACTTCACCCCAGTC	64

16S rDNA gene amplification was conducted using universal primers Y1 (5’-TGGCTCAGAACGAACGCTGGCGGC-3’) and Y3 (5’-TACCTTGTTACGACTTCACCCCAGTC-3’) for all samples with an amplicon size of ~1500 bp [25].

DNA amplification using the listed primers was performed with the PCR conditions with an initial denaturation at 95°C for 10 min, in succession with 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min.This was followed by a final extension at 72°C for 10 min, with a subsequent hold at 4°C for 1 hour. A negative control containing sterile deionized water was included to confirm the validity of the assay.

2.5.3. Agarose gel electrophoresis of PCR product

To validate successful amplification, 10 µl of the PCR product was combined with 2 µl of DNA loading dye (6X) and subjected to electrophoresis alongside a 3 kb marker on a 2% agarose gel containing 4µl of 10 mg/mL ethidium bromide solution. Electrophoresis was conducted at 70V for 90 minutes in 1X TAE buffer. A standard DNA ladder was utilized to determine the size of the amplified 16S rDNA region, and the PCR products were visualized under UV light using a gel documentation system.

2.5.4 Purification of PCR Product

The PCR product amplified with universal 16S rDNA primers Y1 and Y3 with amplicon size of ~1500 bp of all PGPR samples were carefully excised from the low-melting agarose gel using a sterile scalpel while the gel was placed on a low-intensity UV transilluminator. The gel segment containing the desired fragment was transferred into a sterile microcentrifuge tube. The excised PCR fragments were then processed using a Qiagen Gel Extraction Kit to recover the PCR product from the agarose gel. The purification procedure followed was based on the protocol outlined in the user manual, as described by Sambrook & Russell (2001) [31].

2.5.5 Alignment and phylogenetic analysis

The PCR product was then sent for DNA sequencing at Eurofins Genomics India Private Limited, Bengaluru, India.Partial 16S rRNA gene sequence analysis was performed from the PGPR strain and compared with available nucleotide databases. The 16S rRNA gene sequences of the isolates were aligned and compared with known 16S sequences using theBLAST server (www.ncbi.nlm.nih.gov/BLAST) with the Basic Local Nucleotide Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information. The 16S rRNA gene sequences in the GenBank database were used to find the closest database sequence. The 16S rRNA gene sequences of the isolates were aligned with the sequences in the GenBank database using Clustal W for multiple sequence alignment. The evolutionary history was inferred using the Neighbor-Joining method [32]. The phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA 11.0) [33,34]. Finally, the sequences were submitted to GenBank and assigned accession numbers [35,36].

3. RESULTS AND DISCUSSION

RESULTS

3.1 Morphological Characterization of PGPR

Morphological characterization of rhizobacterial isolates was conducted as described by Swarupa *et al*. in their study [37]. The PGPR strains were inoculated on nutrient agar medium at an incubation temperature of 37°C for 24 hours and then subjected to microscopic examination. The various observations of PGPR strains *viz*., colony size, shape, colour, size, consistency, gram reaction, growth speed and brightness were recorded (Table 2).

All the PGPR were gram-negative except *B. subtilis* which was gram-positive. All the bacteria were rod-shaped. Size was observed large in *P. fluorescens* VNMKV1 and medium in *P. putida* VNMKV1*, B. subtilis* VNMKV1*.* The colour of *P. putida* was creamy, *P. fluorescens* was yellowish and *B. subtilis* was whitish. Consistency of *B. subtilis* wasmucous type and fluid typefor *P. fluorescens*, *P. putida*. All the bacteria showed fast growth speed. Colony morphology for *P. putida, B. subtilis* was opaque whereas *P. fluorescens* was observed with translucent colony morphology.

Table 2: Morphological characterization of efficient PGPR isolates

PGPR isolates	Size	Shape	Colour	Consistency	Gram reaction	Growth speed	Brightness
Pseudomonas fluorescens VNMKV1	Large	Rod	Yellowish	Fluid	-ve	Fast	Translucent
Pseudomonas putida VNMKV1	Medium	Rod	Creamy	Fluid	-ve	Fast	Opaque
Bacillus subtilis VNMKV1	Medium	Rod	Whitish	Mucous	+ve	Fast	opaque

(-ve gram negative; +ve gram positive)

3.2 Biochemical Characterization of PGPR

Each PGPR isolate was evaluated using a range of biochemical assays, including the catalase test, starch hydrolysis assay, gelatin hydrolysis test, citrate utilization assay, indole production test, and tetrazolium test. The results of these biochemical test were recorded (Table 3)**.** All isolates yielded positive results in the starch hydrolysis assay, as the clear zones around colonies were observed after flooding cultures with iodine solution whereas no clear zone was observed in the control plate. In the citrate utilization test, the colour of media

in all the tubes changed from green to blue 24 hr after inoculation while no colour change was observed for control. In the gelatine hydrolysis test, all the tubes that were inoculated with respective bacteria were liquified after ice incubation and the control tube was observed solid. In the catalase test, all isolates released O_2 in the form of foamy bubbles from the colonies, which confirmed the positive activity of catalase, while no foaming was observed in the control. In the indole production test, all the isolates showed positive results as the red colour ring at the top of the broth was observed upon adding 10 drops of Kovac's reagent. In the tetrazolium test, *P. fluorescens* showed a deep red colour while *B. subtilis and P. putida* showed a light red colour.

Table 3: Biochemical characterization of efficient PGPR isolates

PGPR	Starch Hydrolysis Test	Gelatine Hydrolysis test	Catalase Test	Tetrazolium Test	Indole production Test	Citrate Utilization test
Pseudomonas fluorescens VNMKV1	+	+	+	+	+	+
Pseudomonas putida VNMKV1	+	+	+	+	+	+
Bacillus subtilis VNMKV1	+	+	+	+	+	+

(**+** positive result; - negative result;)

3.2.1 Antagonistic activity

The antifungal activity of three PGPR isolates was assessed against *Fusarium oxysporum* under *in vitro* conditions using PDA medium (Figure 1). The isolates demonstrated varying levels of inhibition with percentages ranging from 10.95% to 38.35%. *P. fluorescens*, *P. putida* and *B. subtilis* showed potential antifungal effects against this phytopathogen while control plate had 0% rate of inhibition. In the antifungal assay against *F. oxysporum*, the highest rate of inhibition (38.35%) was observed with *P. fluorescens* VNMKV1 followed by *P. putida* VNMKV1 (30.13%). The lowest inhibition was observed with *B. subtilis* VNMKV1which showed 10.95% inhibition (Figure 2).

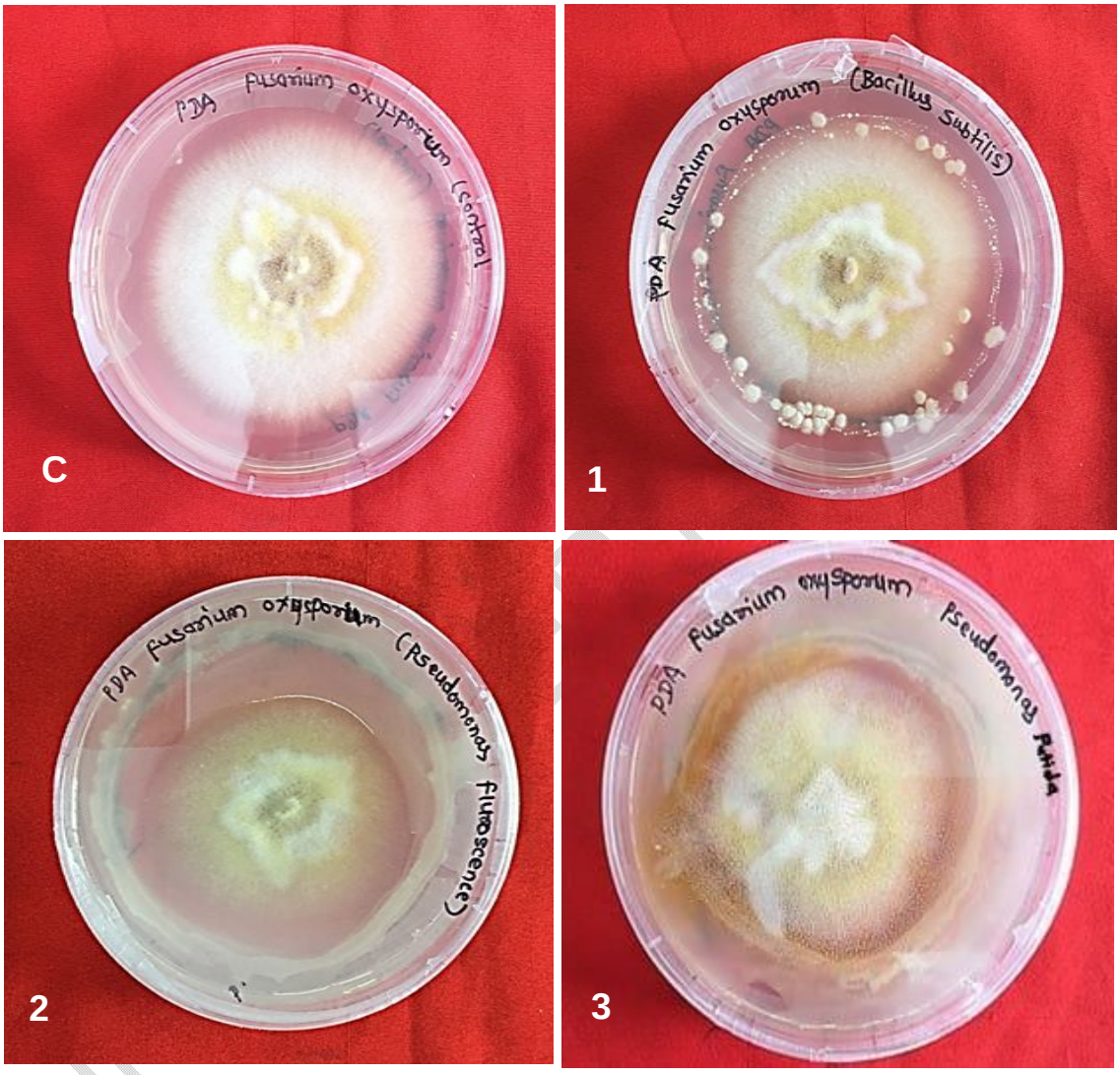


Fig. 1. Antagonistic activity of PGPR against *Fusarium Oxysporum*

C: Control**, 1:** *Bacillus subtilis,* **2.** *Pseudomonas fluorescens,* ***3.*** *Pseudomonas putida*

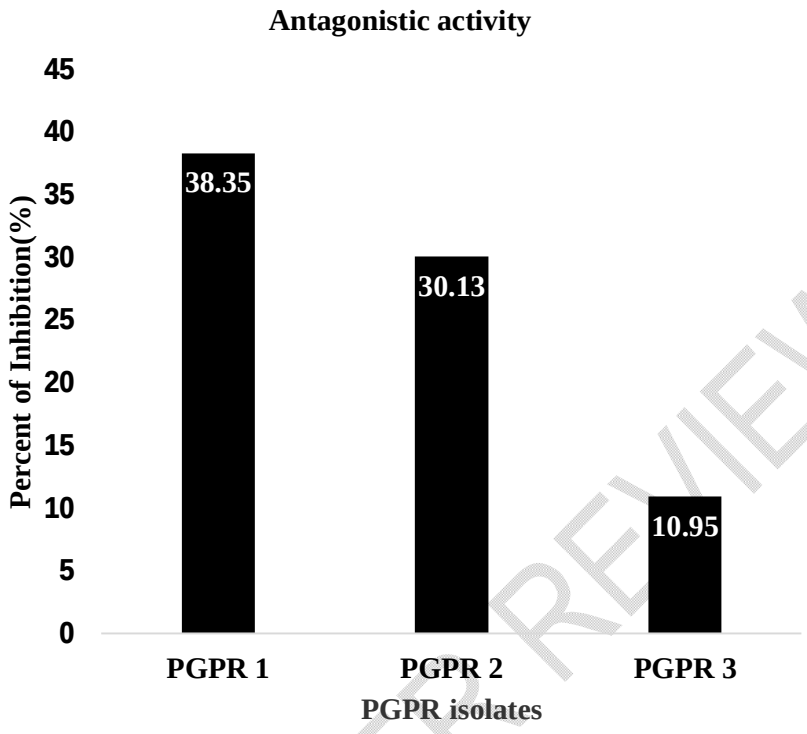


Fig. 2. The antagonistic potential of efficient PGPR isolates on the radial growth of *Fusarium oxysporum*

PGPR 1: *Pseudomonas fluorescens*, **PGPR 2**: *Pseudomonas putida*, **PGPR 3**: *Bacillus subtilis*

3.3 Molecular Characterization of PGPR

DNA was extracted from respective PGPR isolates as the procedure described by Nour *et al*. in their study and quality as well as purity was checked on agarose gel electrophoresis[30]. All the clear bands of DNA were observed.

3.3.1 PCR screening of 16S rDNA gene

PCR amplification of the 16S rDNA gene from three PGPR isolates was carried out using universal primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y3 (5'-TACCTTGTTACGACTTCACCCCAGTC-3'). This primer pair effectively amplified a band of approximately 1500 bp on a 2% agarose gel (Figure 3). This ribosomal region is commonly

utilized for the identification and assessment of diversity among various bacterial groups [38]. The PCR products amplified with universal 16S rDNA primers Y1 and Y3, yielding amplicons of ~1500 bp from all PGPR samples, were subjected to gel extraction using the Qiagen Gel Extraction Kit. The fragments were eluted from the agarose gel according to the protocol outlined by Sambrook & Russell and sent for sequencing to Eurofins Genomics India Private Limited, Bengaluru, India [31].

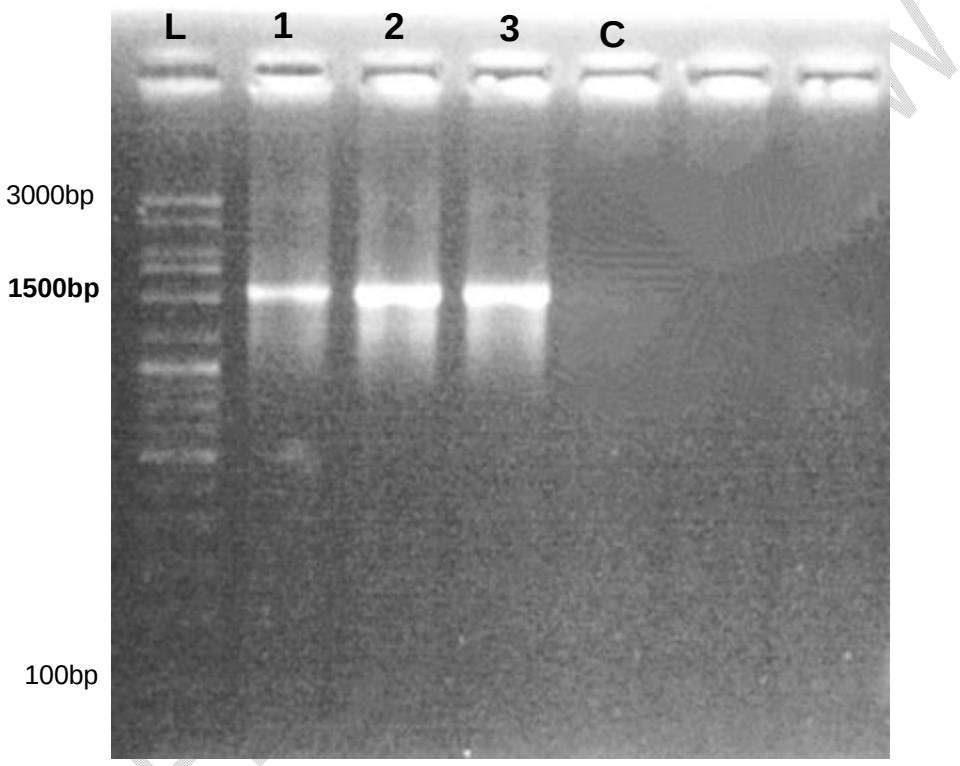


Fig. 3. 16S rRNA gene amplification from PGPR isolates

L - 100 bp to 3000bp, **1.***Pseudomonas fluorescens* **2.** *Pseudomonas putida***3.***Bacillus subtilis*

C- Control

3.3.2 Identification of PGPR isolates using 16S rDNA gene sequencing

The purified PCR amplicon was processed and sequenced ~1500 bp PCR product using the Sanger dideoxy sequencing method. The consensus sequence for the 16S rDNA partial gene was analysed. These gene sequences were compared with the available data in GenBank using the BLAST homology search tool to identify the isolates. As given in Table 4, the Query Cover (QC) for 3 PGPR isolates had a value of 99% to 100%. An E-value of 0.0

signifies the number of alignments with a score equal to or greater than what would be expected to occur randomly in the database.

The PGPR *P. fluorescens* isolate VNMKV 1 showed highest similarity (99.87%) with *P. fluorescens* strain TNUNOM (Accession No. MK157087) with 100% query coverage. The PGPR isolate *P. putida* isolate VNMKV 1 showed highest similarity (100%) to *P. putida* strain CKVF1 (Accession No. OR921377) with 99% query coverage. Similarly, *B. subtilis* showed highest similarity of 99.93% with *B. subtilis* BEST3145 (Accession No. AP024628) with 100% query coverage as given in the Table 4. These sequences were further submitted to the NCBI database and the accession numbers were obtained for *P. fluorescens* (Accession No. PQ274434.1), *P. putida* (Accession No. PQ271576.1) and *B. subtilis* (Accession No. PQ270622.1) as given in the Table 5.

Table 4. The BLAST analysis of 3 PGPR isolates based on 16S rDNA gene sequencing shows related species with similarity and Accession numbers

PGPR Isolate	Related species/strain	Query Coverage	E-value	Similarity (%)	Accession number
Pseudomonas fluorescens VNMKV1	*Pseudomonas fluorescens* strain TNUNOM 16S ribosomal RNA gene, partial sequence	100%	0.0	99.87	MK157087
Pseudomonas putida VNMKV1	*Pseudomonas putida* strain CKVF1 16S ribosomal RNA gene, partial sequence	99%	0.0	100	OR921377
Bacillus subtilis VNMKV1	*Bacillus subtilis* BEST3145 DNA, complete genome	100%	0.0	99.93	AP024628

Table. 5: PGPR isolates with their accession number

PGPR isolates	Accession number
Pseudomonas fluorescens VNMKV1	PQ274434.1
Pseudomonas putida VNMKV1	PQ271576.1
Bacillus subtilis VNMKV1	PQ270622.1

3.3.3 Phylogenetic tree construction

The evolutionary history was inferred by constructing the phylogenetic tree using the Neighbor-Joining method in MEGA11 software. The genetic distance between strains indicated by the scale bar at the bottom of the tree. The horizontal lines (branches) in the

tree denoted the evolutionary distance between the connected nodes, representing genetic divergence.

3.3.3.1 Phylogenetic relationship of *Bacillus subtilis* VNMKV1 based on 16S rDNA gene sequence

The Neighbor-Joining algorithm was applied to infer the evolutionary history of the *B. subtilis* isolate VNMKV1. The construction of the phylogenetic tree described the phylogenetic relationships of the 12 most closely related known strains found with NCBI BLASTn viz., *B. subtilis* BEST3145, *B. subtilis* isolate NRS6121, *B. subtilis* isolate NRS6118, *B. subtilis* strain SRCM102749, *B. subtilis* strain SRCM116268, *B. subtilis* strain BSP110, *B. subtilis* strain UM15, *B. subtilis* strain JD-014, *B. subtilis* strain 50-1, *B. subtilis* strain KA9, *B. subtilis* isolate FELIX, and *B. subtilis* strain YPS, along with the known PGPR isolate *B. subtilis* isolate VNMKV 1 (PQ270622.1), and *Paenibacillusvulneris* strain CCUG, which was taken as an outgroup which provides a reference for rooting the tree and interpreting the direction of evolutionary changes among the *Bacillus subtilis* strains (Figure 4).

The phylogenetic tree revealed the evolutionary relationships among various *B. subtilis* strains, with *P. vulneris* serving as the outgroup, indicating its greater evolutionary distance. At the top of the tree, *B. subtilis* BEST3145 clustered closely with isolates NRS6121 and NRS6118, suggesting minimal evolutionary divergence among them. Strains SRCM102749 and SRCM116268 branched distinctly but remained closely related to the others. Strain BSP110 shared a common ancestor with several strains, including UM15, indicating a close genetic relationship. Strains JD-014, 50-1 and KA9 showed greater genetic divergence but remained in the same clade. *B. subtilis* isolate FELIX and strain YPS exhibited slight divergence from the other strains. The isolate VNMKV 1 (PQ270622.1) clustered closely with strains KA9 and FELIX, indicating their close evolutionary relationship.

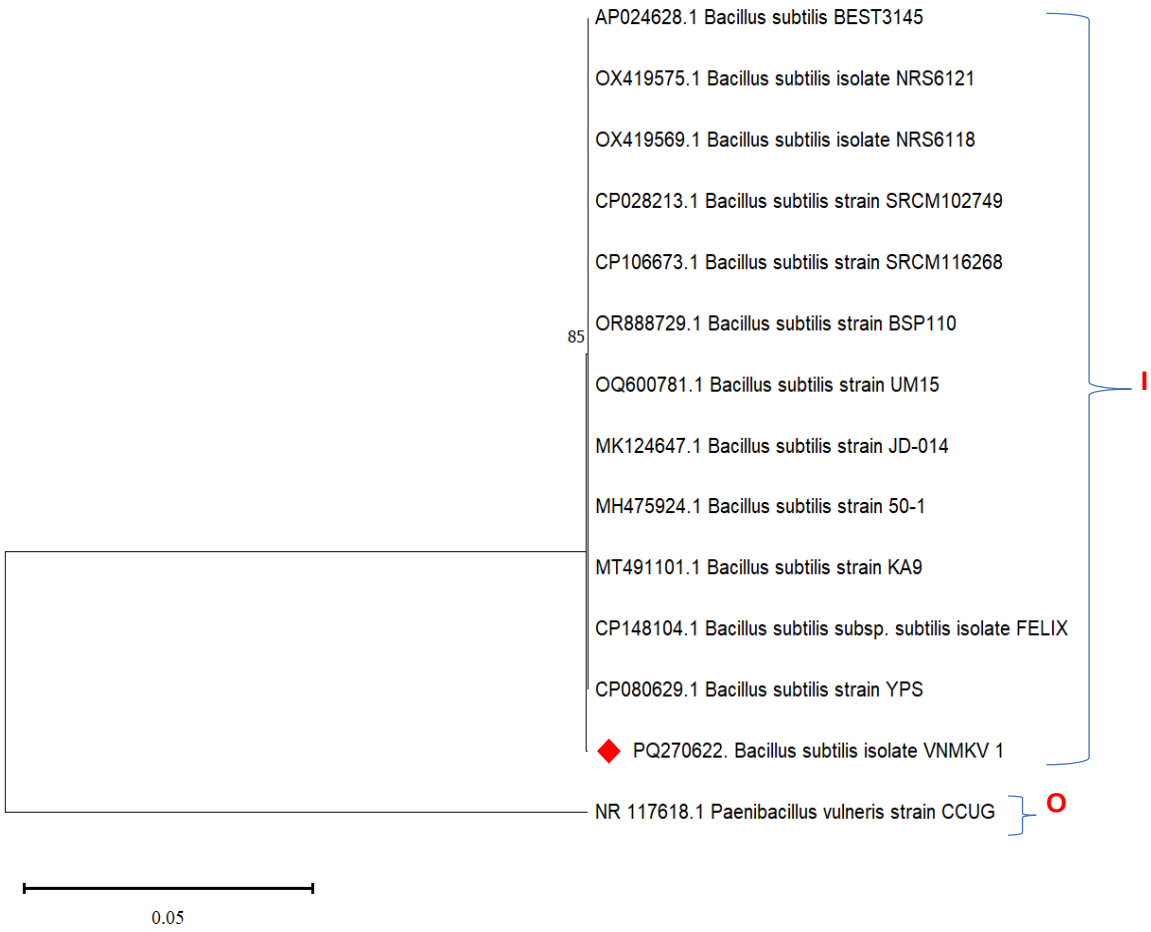


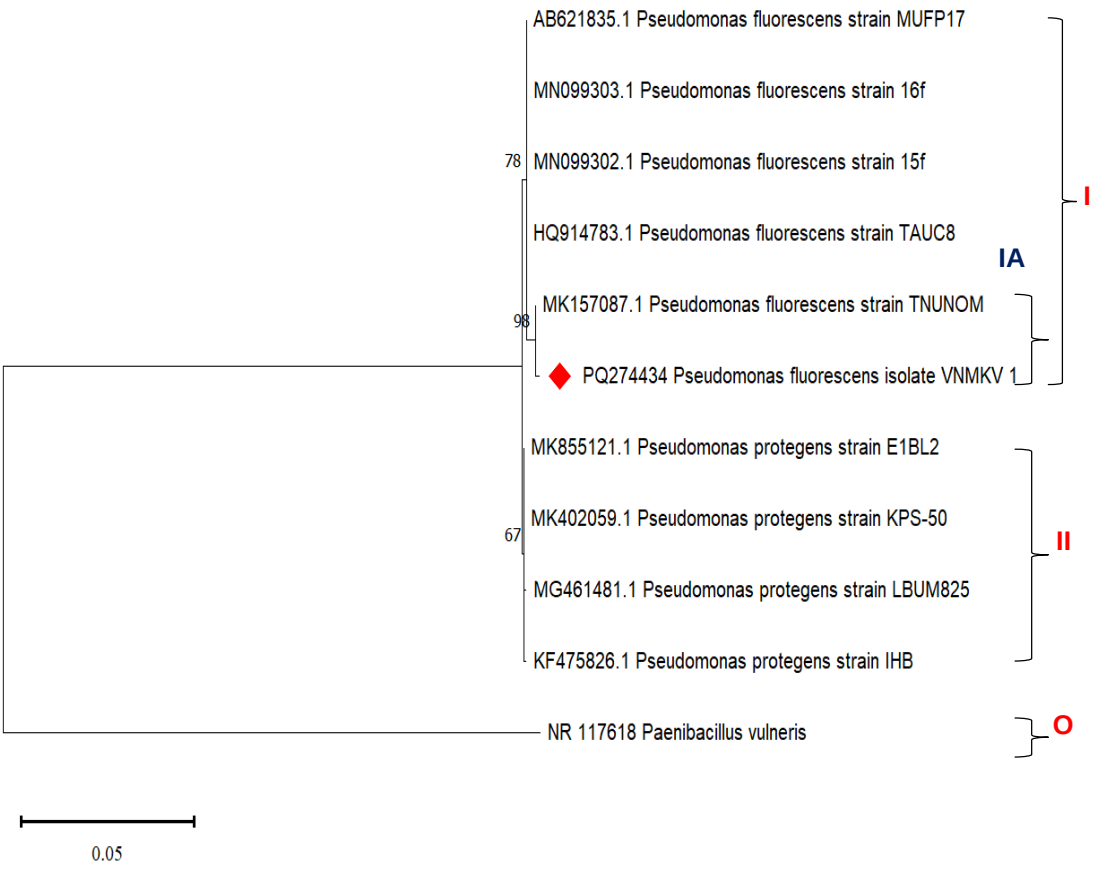
Fig. 4. Phylogenetic tree based on partial 16S rDNA sequences of *Bacillus subtilis* isolate VNMKV 1 with maximum related strain sequences obtained from NCBI BLAST with bootstrap probability values (1000 replications).

3.3.3.2 Phylogenetic relationship of *Pseudomonas fluorescens* VNMKV1 based on 16S rDNA gene sequence

The phylogenetic tree of *Pseudomonas fluorescens*isolate VNMKV1 described the phylogenetic relationship of the 9 most closely related known strains found with NCBI BLASTn software. This included, *Pseudomonas fluorescens* strain MUFP17, *Pseudomonas fluorescens* strain 16f, *Pseudomonas fluorescens* strain 15f, *Pseudomonas fluorescens* strain TAUC8, *Pseudomonas fluorescens* strain TNUNOM, *P. protegens* strain E1BL2, *P.*

*protegens*strain KPS-50, *P. protegens* strain, *P. protegens* strain IHB LBUM825 and *Pseudomonas fluorescens* isolate VNMKV 1 (PQ274434.1), with *Paenibacillusvulneris*strain CCUG, which was taken as an outgroup which provides a reference for rooting the tree and interpreting the direction of evolutionary changes among the strains (Figure 5).

Upon construction of the phylogenetic tree, it was revealed that it was divided into two major clades, clade I and II. clade I included, six taxons were observed *Pseudomonas fluorescens* strain MUFP17, *Pseudomonas fluorescens* strain 16f, *Pseudomonas fluorescens* strain 15f, *Pseudomonas fluorescens* strain TNUNOM, *Pseudomonas fluorescens* strain TAUC8, *Pseudomonas fluorescens* isolate VNMKV 1 (PQ274434.1) at 78 bootstrap value and in clade II, four taxons*P. protegens* strain, *P. protegens* strain KPS-50, *P. protegens* stain LBUM825, *P. protegens*stain IHB were observed at 67 bootstrap values. Major clade I was further divided into a subclade at 98 bootstrap values with highly significant results by *P.*



fluorescens strain TNUNOM, with *P. fluorescens* isolate VNMKV 1.

Fig. 5. Phylogenetic tree based on partial 16S rDNA sequences of *Pseudomonas fluorescens* isolate VNMKV 1 with maximum related strain sequences obtained from NCBI BLAST with bootstrap probability values (1000 replications

3.3.3.3 Phylogenetic relationship of *Pseudomonas putida* VNMKV1 based on 16S rDNA gene sequence

The phylogenetic tree of *Pseudomonas putida* isolate VNMKV1 described the phylogenetic relationship of the 9 most closely related known strains found via NCBI BLASTn software viz., *P. putida* strain SAs-14, *P. putida* strain Cl-14, *P. putida* strain M1, *P. putida* strain CKVF1, *P. putida* strain ATCC 12633, *P. putida* strain AFS093952, *P. putida* strain S6, *Pseudomonas* sp. strain Marseille-Q5299, *P. putida* strain SG11, and known PGPR isolate *P. putida* strain VNMKV 1 (PQ271576.1)and *Paenibacillusvulneris* strain CCUG 53270 which was taken as an outgroup which provided a reference for rooting the tree and interpreting the direction of evolutionary changes among the strains (Figure 6).

The phylogenetic analysis indicated that the tree was divided into two primary clades, Clade I and Clade II. Within Clade I, at 97 bootstrap value 7 taxons were observed *Pseudomonas putida* isolate VNMKV1, *Pseudomonas putida* strain Sas-14, *Pseudomonas putida* strain Cl-14, *Pseudomonas putida* strain M1, *Pseudomonas putida* strain CKVF1, *Pseudomonas putida* strain ATCC 12633, *Pseudomonas putida* strain AFS093952 in clade I, suggesting their close evolutionary relationship. In clade II, three taxons were observed at 83 bootstrap values which were *Pseudomonas putida* strain S6, *Pseudomonas sp.* strain Marseille-Q5299 and *P. putida* strain SG11 showing the highest similarity among them. Clade I also included one subclade at 100 bootstrap value at node which included *P. putida* strain CKVF1, *P. putida* strain ATCC 12633 showing the highest similarity with the PGPR isolate *P. putida* strain VNMKV 1 (PQ271576.1) which are closely related with each other. In clade I, *P. putida* strain Cl-14, *P. putida* strain M1 shows a node at 60 bootstrap values. *Pseudomonas* sp. strain Marseille-Q5299 this species was more distantly related to the *P. putida* and *P. striata* clusters. *Paenibacillusvulneris* represented by the strain CCUG 53270, was the most distantly related organism on the tree which was an outgroup.

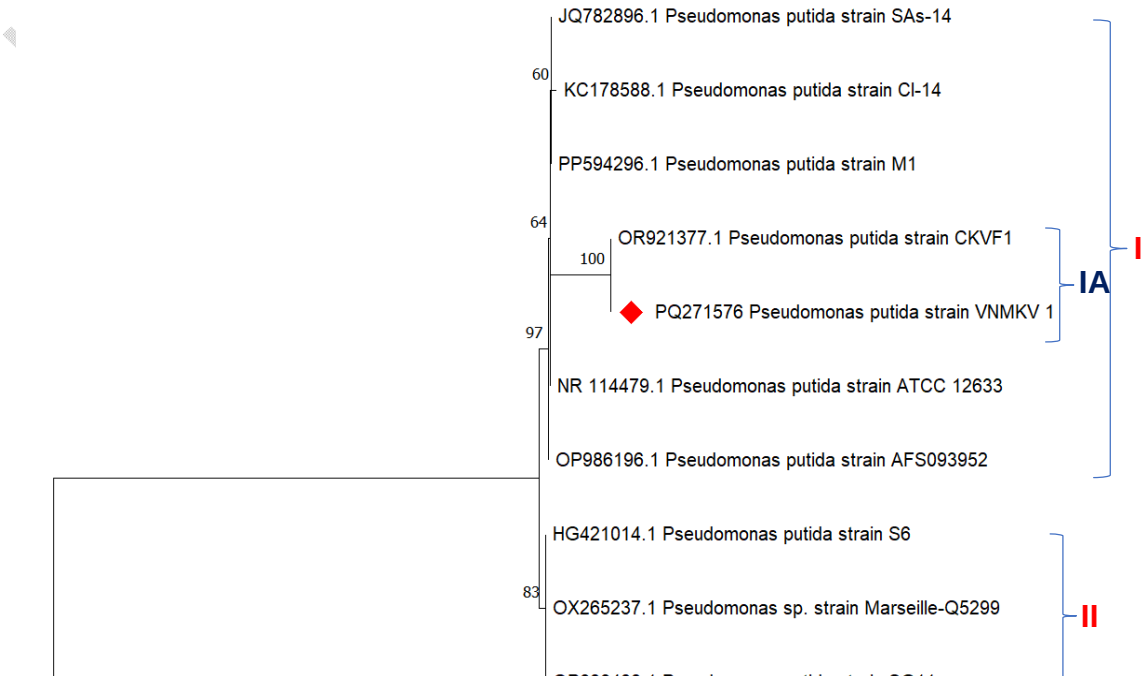


Fig. 6. Phylogenetic tree based on partial 16S rDNA sequences of *Pseudomonas putida* isolate VNMKV 1 with maximum related strain sequences obtained from NCBI BLAST with bootstrap probability values (1000 replications).

DISCUSSION

The adverse effects of various pesticides in agricultural practices have become harmful to humans and the environment. Therefore, there is a need to strengthen research on new developments in biological and environmentally friendly approaches, such as using rhizobacteria to promote plant growth and enhance plant resistance to environmental stress and plant pathogens [39]. Plant growth-promoting rhizobacteria live on plant roots, promoting growth and limiting the population of competitively harmful soil microorganisms [40,41].

The color of colony, growth pattern, fluorescence and cell shape of various isolates of *P. fluorescens* were studied [42].In this study isolates *P. fluorescens*, *P. putida* and *B. subtilis* from the rhizosphere of soybean plants were used. Gram-negative, rod-shaped, motile, aerobic, catalase-positive and oxidation-positive were the characteristics that led to the identification of the *Pseudomonas* genus [22]. Divyanshu *et al*. conducted the morphological characterization of PGPR isolates revealing rod-shaped bacteria, displaying white and yellow pigmentation, with both translucent and opaque appearances. The isolates included both Gram-positive and Gram-negative bacteria, as determined by Gram staining [43].Tatung and Deb did morphological studies of the bacterial isolates of *Pseudomonas putida* and *Pseudomonas monteilii* such as color-off-white, transparency-opaque, colony-flat, margin-filamentous, and form-round [44].*Bacillus subtilis* is a gram-positive, endospore-forming bacteria that plays a major role in biocontrol and PGPR activities. The morphological

characteristics of*Bacillus subtilis* isolates were observed to be Gram-positive, rod-shaped, and capable of forming endospores. The colonies appeared cream-colored to white, with round or irregular shapes and exhibited jagged or serrated edges [45].

Various biochemical assays were performed to confirm the identity of *Pseudomonas* species. Positive results were obtained for gelatine liquefaction, H_2S production, starch hydrolysis, the catalase test, the KOH test, and the acid and gas production test, confirming the isolate as Gram-negative, Similar findings have been reported by several researchers. The isolates of *Pseudomonas fluorescens* and *Pseudomonas putida* exhibited comparable outcomes in Gram staining, starch hydrolysis, gelatine liquefaction, the catalase test, oxidase test, and tetrazolium assay. Saravanan et al. reported that the biochemical profiling of *Pseudomonas* revealed all ten isolates tested positive for catalase, amylase, gelatinase, and siderophore production [46]. Tatung and Deb conducted similar biochemical analysis and observed that isolates were gram negative, catalase test positive, citrate utilization positive, methyl red test negative and starch hydrolysis test positive [44].

Pseudomonas fluorescens, *Pseudomonas putida* and *Bacillus subtilis* isolates could be isolated from the rhizosphere of soybean plant which are known for antagonism toward the soilborne pathogenic fungi. The dual culture method is a commonly employed assay to detect antagonistic bacteria against pathogenic fungi. This technique helps determine whether the isolate can inhibit the growth of pathogenic fungi. The antagonism properties of the isolates indicated their potential to be used as biological control agents for the management of plant fungal diseases. The presence and diversity of these isolates in the rhizosphere could aid in the suppression of soilborne fungal diseases. These plant growth-promoting rhizobacteria isolates present a promising potential for use in biocontrol strategies aimed at managing soilborne pathogenic fungi. Comparable studies have also documented varying outcomes in the suppression of diseases. Boer *et al*. discovered that *Pseudomonas putida* WCS-358 and *P. putida* strain RE8 inhibited the growth of *Fusarium* sp.The disease suppression 30% was observed with treatment of *P. putida* strain RE8, whereas the disease suppression was significantly enhanced to 50% When the two *Pseudomonas putida* strains, WCS-358 and RE8, were combined and introduced into the soil together [47]. Sinha and Jee carried out a study where in vitro screening of rhizobacterial strains AN12, AN15, AN17, and AN18 demonstrated the ability to inhibit *Fusarium oxysporum*, with the highest growth inhibition reaching 58.53% [48].

The molecular characterization of the PGPR isolates analyzed in this study is essential for accurate identification of the bacterial isolates, providing key insights into the potential novelty of the PGPR [49]. The similarity of the bacterial isolates examined in this study complied with the standards and requirements set by the National Centre for Biotechnology

Information (NCBI) for depositing consensus sequences in the NCBI GenBank, facilitating the assignment of an accession number.

A molecular investigation of three isolates i.e. *P. fluorescens* isolate VNMKV1, *P. putida* isolate VNMKV1 and *B. subtilis* isolate VNMKV1 revealed the taxonomy that belong to *P. fluorescens* strain TNUNOM (99.97%), *P. putida* strain CKVF1 (100%) and *B. subtilis* BEST3145(99.93%), correspondingly, derived from phylogenetic analysis and the closest sequence similarity matches, with strong support indicated by a high bootstrap value. In similar study, rhizobacterial strains were identified by 16S rRNA gene sequences. BLAST search tool resulted in the identification of *Bacillus subtilis* based on found similarity. The accession numbers were retrieved from NCBI of the respected rhizobacterial strain.[48]Santosa*et al.* also identified *Bacillus subtilis*, and *Pseudomonas aeruginosa*, by amplifying the 16S rRNA gene followed by sequencing, BLAST analysis and further conducted phylogenetic analysis with available sequences [50].

4. CONCLUSION

Three isolates i.e. *P. fluorescens* isolate VNMKV1, *P. putida* isolate VNMKV1 and *B. subtilis* isolate VNMKV1 were isolated and identified based on morphological, biochemical and molecular characterizations. Morphologically, all isolates were Gram-negative except, *B. subtilis* being Gram-positive and rod-shaped. Colonially, they appeared yellow, cream to white coloured, with medium to large colony size, and also had mucous and fluid-like consistency with rapid growth rates. opaque to translucent brightness. Additionally, in the biochemical characterization, all isolates exhibited positive results for the catalase assay, starch degradation assay, gelatine liquefaction test, citrate utilization assay, indole production test, and tetrazolium reduction test. The antagonistic activity of PGPR isolates against *Fusarium oxysporum*demonstrated significant biocontrol potential. *Pseudomonas fluorescens* exhibited the highest inhibition (38.35%) followed by *Pseudomonas putida* (30.13%) and *Bacillus subtilis* (10.95%). In molecular characterization, Three PGPR isolates were identified using universal primers Y1 and Y3, with a monomorphic ~1500 bp band of the 16S rDNA region. BLAST analysis of the obtained sequence revealed that PGPR isolates *Pseudomonas fluorescens* isolate VNMKV 1 showed 99.87% similarity with *P. fluorescens* strain TNUNOM, *Pseudomonas putida* isolate VNMKV 1 showed 100% similarity to *P. putida* strain CKVF1. *Bacillus subtilis* isolate VNMKV 1 had 99.93% similarity with *B. subtilis* BEST3145. The study successfully contributed novel sequences to the NCBI database, providing accession numbers for all PGPR as *Pseudomonas fluorescens* isolate VNMKV 1 (PQ274434.1), *Pseudomonas putida* isolate VNMKV 1 (PQ271576.1), *Bacillus subtilis* isolate VNMKV 1 (PQ270622.1). Phylogenetic analysis of the 16S rDNA sequences

revealed the phylogenetic relationships of the isolates with their respective strains with high bootstrap values.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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