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Klebsiella pneumoniae VRE36 as a PGPR isolated from *Saccharum officinarum* cultivar Co99004

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ABSTRACT

Klebsiella species known to exhibit important PGP traits like solubilization of phosphate, phytohormone production and good germination potential. In present study, based on the Phosphate solubilization and IAA production bacterial strain VRE36 was selected among the isolates collected from different sugarcane cultivar rhizosphere growing near Bardoli area for characterization and molecular identification through 16S rRNA gene sequence, which confirms the isolate as *Klebsiella pneumoniae*. The phosphate solubilization index of the isolate recorded was 3.9 and quantitative estimation reveals 17.4±1.78µg/ml release of phosphate in NBRIP broth. The high amount of IAA produced was 45.32±2.46µg/ml after 96 hour incubation at 37 ℃. In seed germination assay with *V radiate*, *C tetragonoloba* and *V unguiculata*, treatments with bacteria were supported good plant height, dry weight and fresh weight when compared with controls. Maximum percentage of germination was recorded in *V radiate* (97.78%). The improved seedling parameters of the inoculated crop seeds indicated the potential of this isolate to be used in a bio-fertilizer formulation for sustainable production.

1. INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate (P) solubilization, siderophore production, biological nitrogen fixation and Indole acetic acid (IAA) production [1]. The potentiality of PGPR in agriculture to achieve higher yields is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. The dependence on chemical is associated with problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities that otherwise support crop production. Phosphorous is one of the major micronutrient require by the plant for growth. However, Continues application of phosphorous fertilizer may result in negative impacts soil in terms of accumulation due to its fixation and precipitation behaviour which lower the efficiency of added P. It has been reported that more than 80% of applied P in soil precipitates in the presence of metal ion complexes such as Ca^{2+}, Fe^{3+} and Al^{3+} and remains in soil in insoluble mineral form [2]. Furthermore the prices of P fertilizer raised several fold higher in last few years have made it most expensive and not-affordable to poor farmers. The introduction of PGPR with phosphate solubilization activity can be viable and sustainable option for the removal of unutilized insoluble phosphate from soil by making them available for plant. Investigation of modes of action of phosphate solubilising bacteria (PSB) are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizer [3]. Due to constant liberation of nutrients from plant roots, soil microbes are found to dominate the niche and their by helps in plant growth promotion under various mechanisms [4]. PSB had shown an effective role in growth promotion of plants by dissolving inorganic insoluble phosphate and converting the phosphorus in a form available to the plants [5]. Most PSB are highly specific to a host plant, and host-specificity in colonization is an important parameter for the preparation of biofertilizer [6]. However, some PSB have a rather wide host range. *Pseudomonas sp*. dominated in the rhizosphere of maize, soya bean, oat, and wheat, and *Berkholderia sp.* is being used as a biofertilizer in several important crops [7-10]. A single microbe can be used for several crops, especially when using a nonspecific genus like *klebsiella*. As sugarcane is a long duration crop and faces many biotic and abiotic stresses during growth phase, the PGPR found in sugarcane root zone may be potent and useful for application to other crops

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Keeping in mind the objectives of this study were (i) to isolate PGPR from sugarcane rhizosphere under *in vitro* conditions and to select the potent isolate on the basis of phosphate solubilization (ii) to analyse biochemical properties, antibiotic susceptibility, IAA production and 16S rRNA sequence (iii) to assesses the PGP potency of isolate by seed germination assay *in vivo* on cereal crops in a pot experiment.

2. MATERIAL AND METHODS

2.1. Bacterial strain isolation and screening

A rhizospheric soil samples from sugarcane was used for the isolation of PGPR. Samples were added into a flask containing 90ml mL sterile PBS and then made serial dilutions up to 10^7. 100 µl from last three dilutions was taken as an inoculum in the plate and melted nutrient agar (NA) media was poured in to it. Then the inoculated plates were incubated in an incubator at 37 °C for 24 hours. After collection of maximum number of diverse organisms, initial screening was performed for phosphate (P) solubilization on Pikovskya medium and IAA production in LB broth with 0.1% tryptophan. The best phosphate solubilising bacteria (PSB) with ability to produce IAA was selected for further study.

2.2. Morphological and Biochemical characterization

Isolate was characterized for Gram staining, morphology and biochemical characters such as catalase, oxidase, and carbohydrates fermentation starch hydrolysis capability, catalase activity, Methyl red (MR), citrate utilization, phenyl alenine deaminase, Gelatin Hydrolysis, MR (Methyl Red), Triple Sugar Iron Agar (TSIA), Urease, Voges Proskauer (VP) etc. [11]. All incubations of the biochemical tests were done at 37°C.

2.3. Antibiotic Susceptibility test

To evaluate the susceptibility of *K pneumoniae* VRE36 to different antibiotics overnight grown 1ml of culture was inoculated into sterile 20ml melted nutrient agar and poured into sterile 9Cm Petri plates. After the media got solidified HiMedia hexa antibiotic disc were placed on the surface followed by overnight incubation at 37°C and clear zone of inhibition was recorded.

2.4. Phosphate solubilization activity

To determine the phosphate solubilization, Pikovskaya's agar [12] plates were spot inoculated with *K pneumoniae* culture and incubated at 30°C for 4 days. Appearance of clear halos around bacterial colony indicated the phosphate solubilisation ability and solubization index was measured [13].

Phosphate solubilization index = (colony diameter + halo zone) / colony diameter

Quantitative estimation of soluble phosphate in broth was carried out using Erlenmeyer flasks (150 ml) containing 25 ml of NBRIP medium which contained per litre: glucose - 10gm; $MgCl_2.6H_2O$ - 5gm; $MgSO_4.7H_2O$ - 0.25gm; KCl - 0.2gm; $(NH_4)_2SO_4$ - 0.1gm. Autoclaved broth was inoculated in triplicate with the bacterial strain K *pneumoniae* and uninoculated medium served as control. The flasks were incubated for 96h at 30°C on an incubator shaker at 180 rpm. The cultures were harvested by centrifugation at 8000 rpm for 15 min. Phosphorus in the cell free culture supernatant was determined. For this, 1 ml of the supernatant was taken, 2.5 ml of Barton's reagent was added and volume was made up to 50 ml with distilled water. After 15 minutes, the intensity of yellow colour was read on spectrophotometer at 430 nm and the amount of P solubilised was extrapolated from the standard curve.

2.5. Indole acetic acid (IAA) production

Production of IAA by the *K pneumoniae* was detected according to the modified method described by Bric [14]. Twenty four hour grown culture was inoculated into 10 ml sterile LB amended with 0.1% tryptophan. After incubation at 37 °C for 96 h IAA produced was determined in culture supernatant (1ml) using Salkowski reagent (2ml) and the amount of IAA produced was extrapolated from the standard curve. Optical density was measured at 530 nm on spectrophotometer (Shimadzu).

2.6. Seed germination assay

K pneumoniae VRE36 inocula was prepared by inoculation of single colony into nutrient broth and kept in an orbital shaker (150 rpm) for 24 h. Culture was centrifuged in 15 ml sterile plastic tubes at 6000 rpm for 15 min and pellets were re-suspended in sterile distilled water (SDW) to obtain a final concentration of 10^8 colony forming units (CFU/ml). Seeds of Cluster bean, green gram and black eyed bean were sterilized by dipping in 2% NaOCl solution for 10 min and then washed three times with SDW. Seeds were dipped into the inocula (10^8 CFU/ml) for 30 min and dried under laminar airflow condition and planted into the pot containing 1 kg four time autoclaved soil (pH 7.53, N-419.2 mg/kg, P -8.26 mg/kg, K-156.69 mg/kg, Carbon - 0.69%, EC- 4.38 ds/m). Seed treated with SDW were served as control. Experiments were performed in a completely randomized block design and growth parameters were measured after 5 days.

2.7. Molecular identification and phylogeny

The genomic DNA was isolated according to Sambrook [15]. Amplification of 16S rRNA gene of BPR7 was carried out by PCR (Eppendorf) using universal eubacterial primer set 8F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'- CGG TTA CCT TGT TAC GAC TT-3'. The total PCR reaction mixture was 50µl comprising 200 mM dNTPs, 50mM each primer, 1X PCR buffer, 2U Taq polymerase, and 100ng genomic DNA. The thermocycling conditions involved an initial denaturation at 94 ◦C for 4 min, followed by 35 cycles of 94 ◦C for 1 min, 52 ◦C for 1 min, and 72 ◦C for 2 min and final extension at 72 ◦C for 8 min. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Similarity of 16S

rRNA gene sequence was aligned using BLAST programme of GenBank database (NCBI) and aligned to their nearest neighbours. The evolutionary distances among *K pneumoniae* isolates and phylogenetic tree was constructed using MEGA 6.0 software and Kimura's two-parameter model, after aligning the sequences with ClustalW.

3. RESULT AND DISCUSSION

PGPR are free living soil bacteria that aggressively colonize the plant roots and when applied to the seeds or crops they enhance the growth and yield of the plant [16]. The presence study carried out to characterize the potential of sugarcane PGPR *K pneumoniae* to support the growth of plants by its biochemical activity. In total 108 organisms were isolated on nutrient agar plate and on the basis of P solubilization activity and IAA production, strain VRE36 was selected for further work.

3.1. Morphological and Biochemical characterization

Morphological characterization of the isolate based on Gram test revealed that the bacterium was gram negative and rod shaped. Colony characters on Nutrient agar plate shows that isolate was non pigmented, fast growing, having small round shape colony with raised elevation and smooth surface.

It was observed that out of fifteen biochemical test performed, isolate shown positive results for ten tests. The biochemical analysis results are summarized in Table 2.

3.2. Antibiotic Susceptibility test

K pneumoniae VRE36 susceptibility was evaluated against 27 antibiotics and found to be sensitive against 22 antibiotics (Table 3). *K pneumoniae* was resistant to 5 antibiotics namely Ceftazidime, Linezolid, Cloxacillin, Lincomycin, and Cefuroxime.

3.3. Phosphate solubilization activity

Phosphorous is a second major nutrient after nitrogen require for plant growth. The bacteria with good ability to solubilise phosphate should have a good halo zone to colony ratio. This criterion is generally used for preliminary screening of phosphate- solubilising micro-organisms including fungi and bacteria. In present study the phosphate solubilization index of the isolate recorded was 3.9. The ratios for bacteria in this study were much higher than 1.17–2.96 for phosphate-solubilising fungi in asparagus root zone [17]. Islam *et al.* [18] reported the phosphate solubilization index of the rice isolates varied from 1.2 to 6.7. Many species of rhizobacteria including *Bacilli, Pseudomonas* and *Klebsiella* can solubilise insoluble phosphates in the agar assay *in vitro* [19-20]. Quantitative estimation in NBRIP broth reveals 17.4±1.78µg/ml solubilised P (Table 1). This data is in accordance with the Henry et al reported 14.23µg/ml P solubilisation by HB3 strain [21]. Zahid et al reported the P solubilisation potential of 19.2µg/ml and 35.6µg/ml for *Pseudomonas stutzeri* and *Bacillus subtilis* respectively [22]. The activity of PSB is crucial for crop production under condition where P is a limiting factor.

Table 1: Details of Sugarcane Co99004 root rhizosphere isolate VRE36.

Isolate code	identified by 16s rRNA	host cultivar	place of collection	homology	Accession number	IAA production µg/ml	Phosphate Solubilization Index	Phosphate solubilization µg/ml
VRE36	*Klebsiella pneumoniae*	Co99004	Bardoli	91	KX418655	45.32±2.46	3.9	17.4±1.78

Table 2: Biochemical properties of *Klebsiella pneumoniae* VRE36.

Biochemical Test	Result
Glucose	Positive
Lactose	Positive
Maltose	Positive
Mannitol	Positive
Sucrose	Positive
Xylose	Positive
Phenylalanine Deaminase	Negative
Catalase	Positive
Citrate	Positive
Gelatin Hydrolysis	Negative
MR (Methyl Red)	Negative
Oxidase	Negative
TSIA (Triple Sugar Iron Agar)	Negative
Urease	Positive
VP (Voges Proskauer)	Positive

Table 3: Antibiotic susceptibility of *K pneumoniae* VRE36.

Antibiotic Disk	Zone of Inhibition (mm)	Antibiotic Disk	Zone of Inhibition (mm)	Antibiotic Disk	Zone of Inhibition (mm)
Ampicillin 10µg	10	Cefotaxime 30µg	15	Amoxyclav 30µg	11
Ceftriaxone 30µg	16	Amikacin 30µg	22	Clarithromycin 30µg	10
Chloramphenicol 30µg	21	Nitrofurantoin 300µg	13	Cloxacillin 1µg	R
Ciprofloxacin 5µg	27	Netillin 30µg	21	Cephalothin 30µg	10
Co-Trimoxazole 25µg	14	Nalidixic acid 30µg	24	Lincomycin 2µg	R
Tetracycline 30µg	25	Vancomycin 30µg	9	Cefuroxime 30µg	R
Cefpodoxime 10µg	9	Oxacillin 1µg	17	TobramycinM 10µg	21
Clavulanic acid 5µg	11	Linezolid 30µg	R	Erythromycin 15µg	28
Ceftazidime 30µg	R	Clindamycin 2µg	10	Gentamicin 10µg	18

3.4. Indole acetic acid (IAA) production

Interaction between microbes and plants is well studied for symbiotic mechanism in which plant releases amino acids, vitamins and other nutrients and microbes metabolize these substances to produce products which influence the growth of the plants. IAA is an acid hormone synthesize by microorganism by various known pathways which may be tryptophan dependent or independent [23]. In soil, the tryptophan may be available in the rhizosphere naturally through root exudates as reported by Beniziri et al [24]. In the present study LB medium was amended with 0.1% tryptophan which act as a precursor for the biosynthesis of IAA. The production of phytohormone IAA is considered as an effective tool for screening beneficial microorganisms as there have been reports suggesting that IAA producing bacteria have profound effect on plant growth [25]. In the presence of tryptophan, the amount of IAA produced was estimated from the standard and recorded 45.32±2.46µg/ml after incubation at 37 °C for 96 h (Table 1). Colour development was first visible within minutes and continued to increase in intensity for a period of 30 min. Similar research report has been documented on *Klebsiella* species for phytohormone production from rhizosphere of sugarcane, soyabean and rice [25, 26-27]. IAA production for *K pneumoniae* in present study was comparable to that reported by other authors for *Klebsiella* species K8 (171.9µg/ml) and K42 (11.12µg/ml) in chemically defined media [28].

3.5. Seed germination assay

The seeds are the reproductive part of the plant which is expected to give rise healthy plant. The oretically, application of PGPR should promote shoot growth and root growth, and, therefore, plant height, fresh weight and dry weight of sterilized soil control should be higher than those of without treatment. In this study, application of K *pneumoniae* supported higher germination rate and other growth parameters (Figure1). Similar studies on *Klebsiella* strains for plant growth promoting traits on wheat plant under axenic conditions suggests that these are promising strains for application in agriculture [28]. In recent study, maximum percentage of germination (GP) was recorded in *V radiate* (97.78%).

The variations in plant (height, dry weight and fresh weight) among the control and treated seeds were significant (Table 4).

The increased rate of seed germination and seedling parameters in PGPR treated seeds as compare to uninoculated seeds may be due to the release of growth hormone IAA [29]. In case of *C tetragonoloba*, plant heights increased from 12.10 cm (control) to 17.03 (treatment) were observed (Table 4). As with plant height, seed treatment also had the highest plant dry weight (0.29 g), which was not significantly different from control (0.16 g). Treated *V radiata* seeds had shown the highest plant height (23.43 cm) as compared to control (15.97 cm). Similar observations were made in the case of growth parameters for *V unguiculata.*

In all pot trial, treatments with bacteria were significantly higher than control. As the isolate in this study produced higher IAA and have phosphate solubilization activity, it might have directly or indirectly influenced the seed germination and plant growth.

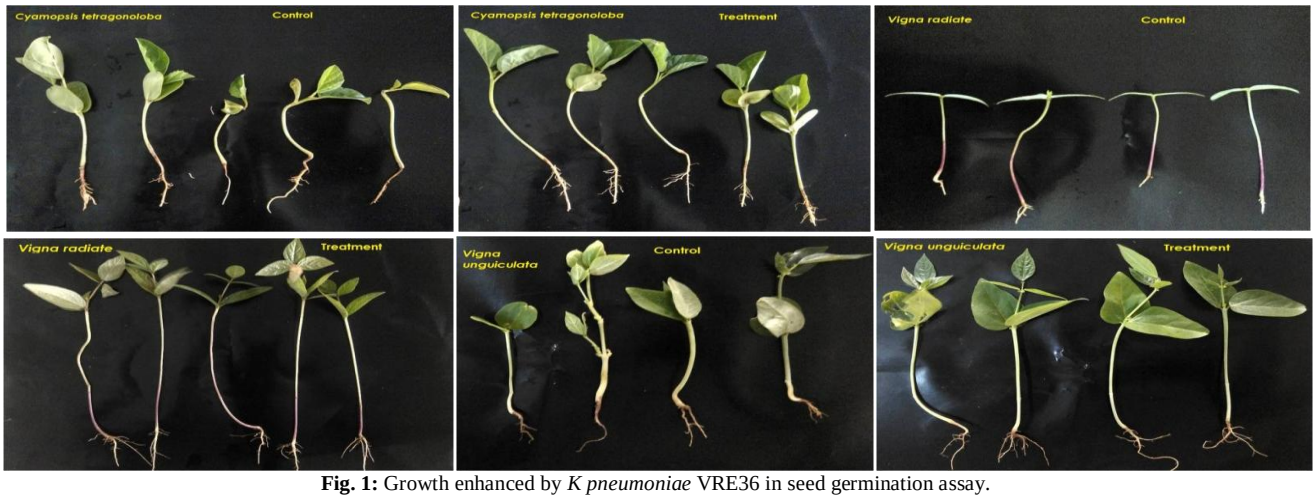

Fig. 1: Growth enhanced by *K pneumoniae* VRE36 in seed germination assay.

Table 4: Effects *K pneumoniae* VRE36 on plant growth parameters in seed germination assay. Data represents Mean (n=10) ± Standard Deviation. Germination percentage (GP), Plant Height (PH), Leaf width (LW), Leaf height (LH), Shoot Length (SL), Root length (RL), Fresh Weight of plant (FWP), Dry weight of plant (DWP).

Seeds		GP	PH(Cm)	LW(Cm)	LH (Cm)	SL(Cm)	RL(Cm)	FWP(Gm)	DWP(Gm)
C tetragonoloba	Control	79.44±8.22	12.10±1.91	2.03±0.35	3.30±0.66	3.97±0.55	3.77±0.55	0.56±0.06	0.16±0.03
	Treated	93.89±2.55	17.03±1.50	3.23±0.23	3.50±0.20	8.17±0.96	4.97±0.45	0.84±0.11	0.29±0.03
V radiata	Control	65.56±8.55	15.97±0.55	1.36±0.15	3.30±0.92	10±1.10	2.57±0.65	0.25±0.03	0.12±0.02
	Treated	97.78±2.55	23.43±1.36	1.59±0.19	4.93±1.20	16.20±0.95	3.97±0.65	0.54±0.08	0.21±0.03
V unguiculata	Control	75.56±7.52	10.82±0.63	2.07±0.21	2.68±0.24	6.16±0.19	2.63±0.18	0.94±0.02	0.28±0.02
	Treated	96.11±0.96	22.58±2.70	3.52±0.09	5.27±0.57	10.34±0.60	5.57±0.35	1.41±0.20	0.65±0.02

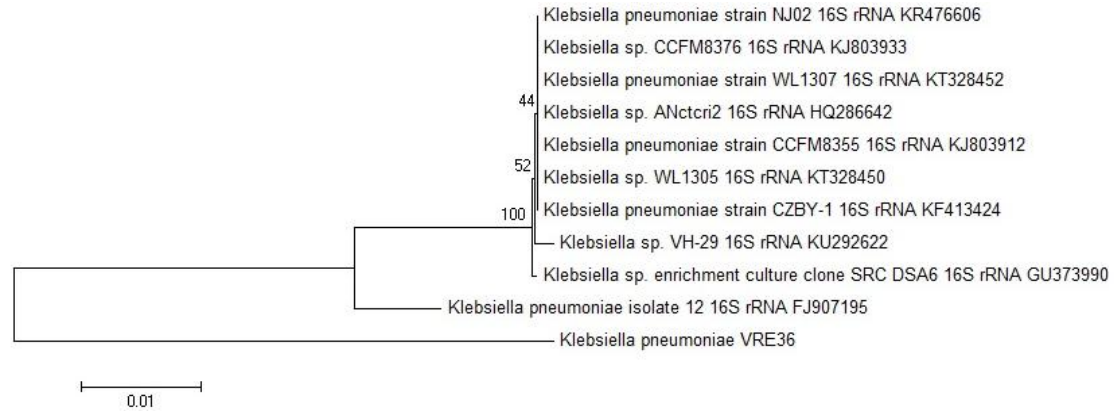

Fig. 2: A neighbor-joining tree derived from sequences of 16S rRNA region. Numbers on nodes represent bootstrap values (%) from 1000 replicates. A phylogenetic tree was constructed using MEGA 6.0 with kimura-two parameter model. Bar represents 0.01 substitutions per site.

3.6. Molecular identification and phylogeny

The isolate were later identified for their species using partial 16S rDNA sequencing method. BLAST results confirmed that the isolate homology was identical to *K pneumoniae* isolate 12 with 91% of homology (FJ907195.1). The 16S rDNA gene sequences of *K. pneumoniae* VRE36 determined in this study were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession number KX418655. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length 0.09827708 is shown (Figure 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1084 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0. Phylogenetic tree of PSB *Klebsiella* strain VRE36 revealed that the strain is presented on separate clade, indicating that these strains of *Klebsiella* was of different phylotypes. Isolation source of *Klebsiella strains* selected NCBI to construct the tree was different form our isolate like Hami-melon juice (FJ907195), dental caries (GU373990), rhizosphere region of Rockwell plant (HQ286642) so that may be the reason for our sequence VRE36 does not fall in any cluster.

4. CONCLUSION

The research met our objective in identifying the bacteria from Co99004 as a most promising and worth exploring in further studies to develop bio-fertilizer with good ability to solubilise phosphate and production of phytohormone IAA. Although isolate *K pneumoniae* supported good germination and plant growth tested, further investigations in details are also required to confirm their ability in field.

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