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Isolation, identification and screening of phosphate solubilizing bacteria from the *Piper betle* fields of Guntur District, Andhra Pradesh, India

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Key words: *Piper betle*, Rhizosphere soil, *Pseudomonas fluorescens* ASN-2, Phosphate solubilization

Abstract

A study was designed to screen the effective phosphate solubilizing rhizobacteria from rhizosphere of Betle vine plants (*Piper betle*). Soil samples collected from agriculture fields of *Piper betle* of Guntur district (Nutakki, Revendrapadu), Andhra Pradesh, India and they were employed for isolation of rhizobacteria by using serial dilution plate technique. To isolate rhizobacteria, two media such as Nutrient agar and Kings' B agar were employed. Total 15 rhizobacterial strains were isolated from the rhizosphere samples and designated them as ASN-1 to ASN-15 and evaluated for their phosphate solubilizing ability. Among them, ASN-2 showed phosphate solubilisation efficiency. The maximum phosphate solubilization halo zone was recorded as 30 mm on Pikovskaya's agar plates after 96h of incubation at 28±2^{0}C whereas in case of broth, the strain exhibited highest tricalcium phosphate (TCP) solubilization as 197.32µg/ml. Characterization of the strain was performed based on cultural, morphological and genomic characteristics using 16s r RNA sequencing and identified as *Pseudomonas fluorescens* ASN-2 (GenBank accession number: MW537707). The study suggested that rhizobacteria (*Pseudomonas*) from rhizosphere of vegetable crop Piper betel of Andhra Pradesh (Nutakki, Revendrapadu) serve as a source for potential phosphate solubilizing property.

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Introduction

The Betel vine plant (*Piper betle* L.) is a climber with spicy, heart-shaped leaves, widely cultivated in South-Eastern Asian countries and widely used for both traditional and medicinal practices (Tallapragada and Seshachala, 2012). The rhizospheric soil of the Betel vine plant has diverse PGPR microorganisms, some of which have potent phosphate-solubilizing capacities.

Plant growth promoting rhizobacteria (PGPR) are the soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development *via* production and secretion of various regulatory chemicals in the vicinity of rhizosphere. Generally, PGPR facilitate the plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of bio control agents (Khan *et al*., 2009; Rodriguez and Fraga, 1999).

Phosphorus (P), is one of the major, essential macronutrient required for the development of the plants (Inchure and Vedpathak, 2021) and also it is one of the most limiting factors in crop production in many kinds of soils. The concentration of soluble phosphorus (P) in many soils is usually very low compared to other mineral nutrients. Phosphorus is available in micro-molar quantities or less. Rhizospheric phosphate solubilizing bacteria (PSB) are capable of solubilizing insoluble or inorganic phosphates into soluble organic forms. Such PSMs are known to be abundant in the rhizospheric soils of various plants especially in betel vine plant. Phosphorus biofertilizers in the form of micro-organisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Mahantesh and Patil, 2011). Individual or co-inoculation of PSB with other groups of microorganisms enhanced the plant growth by increasing the efficiency of biological nitrogen fixation or the availability of other trace elements and by the production of plant growth promoting (PGP) substances (Bychkova *et al.*, 2022).

Several varieties of phosphate-solubilizing microorganisms (PSMs) have been isolated from the rhizospheric soils of crops. Of these, 20%-40% are culturable soil microorganisms. A majority of the isolated organisms are bacterial organisms, although several fungi are also known to solubilize phosphates (Bhattacharyya and Jha, 2012). One of the bacterial genera having the potential to produce bioactive compounds against pathogens is *Pseudomonas*. The *Pseudomonas* (g-Proteobacteria) are non-sporulating rods with Gram negative reaction. Fluorescent *Pseudomonas* sp. are effective phosphate solubilizers which directly or indirectly promote the growth of plants by increasing the concentration of available nutrients and exhibited antibiosis through production of a wide spectrum of antimicrobial components against pathogenic bacteria and fungi (Kshetri *et al*., 2015). Pseudomonades usually produced several metabolites from different groups such as 2,4-diacetylphloroglucinol, 2-acetamidophenol, hydrogen cyanide, indoles, phenazines, phenazine-1 carboxylic acid, pseudotrienic acids, pyocyanin, pyoluteorin, pyrrolnitrin, tenzin and viscosinamide (Dave and Patel, 1999; Paul and Sinha, 2017).

In view of its reputing significance in the agriculture sector, the objective of the current study was aimed at isolation and screening of potential phosphate solubilizing bacterial strains for their their phosphate solubilizing capacity isolated from rhizosphere of *Piper betle* of Nutakki and Revendrapadu villages of Guntur district, Andhra Pradesh, India.

Materials and methods

Sample collection of rhizosphere soil

Betel vine (*Piper betle* L.; *Piperaceae*) rhizosphere soil samples were collected from different betel cultivating areas of Guntur district (Nutakki and Revendrapadu), Andhra Pradesh, India. All the soil samples were collected from rhizospheres of 5-10 randomized young and old Betel vine plants. To maintain uniformity, all the samples were taken at a depth of 10-15cm. and pooled together to make the composite sample. The collected samples were aseptically transferred to laboratory and stored them

in refrigerator for further analysis. The soil samples were dried, crushed and used for isolation (Chung *et al*., 2005).

Isolation of phosphate solubilizing rhizobacteria

The 10 grams of air-dried powdered soil was taken into 100 ml sterilized distilled water and shaken for 15 minutes. Serial dilutions were prepared (10^{-1} to 10^{-6}) and 0.1 ml of each dilution were plated on Nutrient agar medium (NAM) and King's B agar medium (KB) amended with antifungal agents like nystatin (25µg/ml^{-1}) and cycloheximide (25µg/ml^{-1}) (King *et al*., 1954; Grant and Holt, 1977; Gulati *et al*., 2008). All the plates were incubated at 28±2 ^{0}C for 24-48h. After incubation, appeared individual bacterial colonies were sub-cultured and observed under UV light for fluorescence observation.

Screening of rhizobacteria for phosphate solubilizing potential

The isolated, suspected bacterial colonies were screened for their phosphate solubilization potential on Pikovskaya's agar (PKA) medium consisting of constituents: glucose: 10g; TCP: 5g; yeast extract:0.5g; $[(NH_4)_2SO_4]$: 0.5g; KCl: 0.2g; NaCl: 0.2g; $MgSO_4$: 0.1g; $FeSO_4$ trace; $MnSO_4$ trace; Agar-agar: 15g; distilled water: 1L; with adjusted pH 7.0±0.2. The isolated strains were point inoculated over PKA medium and incubated for 48h at 28±2 ^{0}C. After incubation period, the culture colonies showing phosphate solubilization or halo zones were confirmed as positive, they were subcultured on PKA slants and preserved them at low temperature for future studies. Phosphate Solubilization Efficiency/index (PSE) of positive strains were measured in terms of diameter of clearance zone including culture growth and colony diameter (Pikovskaya, 1948; Adhikari *et al*., 2021).

PSE= Colony diameter + Halo zone diameter / Colony diameter

Estimation of phosphate solubilisation efficiency

The phosphate solubilisation was quantitatively estimated by inoculating 1ml of ASN-2 suspension into 50ml of Pikovskaya's (PK) broth in a 250ml Erlenmeyer flask. Incubation was followed after inoculation for 5 days at 28±2°C. After incubation period, culture broth was centrifuged at 10,000 rpm for 10 min. soluble phosphate in culture supernatant was estimated spectrophotometricallly (at 600nm) by standard ascorbic acid method (Watanabe and Olsen, 1965). Uncultured broth sample was treated as blank. After incubation of 5days, pH of the broth was recorded. The experiment was carried out in triplicates.

Characterization of the rhizobacterial strain ASN-2

Morphological and biochemical identification

Characterization of the rhizobacterial strain ASN-2 was studied through culturing on NAM plates and incubated at 28±2^{0}C for 2-4days. Morphology of the strain was studied by Gram's staining technique. The stained culture was observed by using compound microscope. Characteristic feature of fluorescent presence was observed under the UV-light. Different biochemical tests such as catalse activity, oxidase activity, Methyl-red (MR) test, Indole production test, citrate utilization activity and Voges-proskauer (VP) reaction were performed to identify the bacteria (Holt *et al*., 1994). Hydrolysis of starch, nitrate reduction and Hydrogen-sulphide production were also tested (Bhatt and Vyas, 2014; Capuccino *et al*., 1992).

Molecular characterization

The phylogenetic relationship of the strain was determined by the analysis of its 16S r RNA gene sequence and the gene sequence of the strain was obtained by using its gene specific primers such as 8F 5^1-AGAGTTTGATCCTGGCTCAG-3^1 and 5^1-GGTTACCTTGTTACGACTT-3^1. Thus the obtained 16S r RNA gene sequence was analysed at NCBI Gen Bank by using BLAST analysis (http://www.ncbi.nlm.nih.gov) (Espinosa *et al*., 2009). The phylogenetic tree was constructed by Maximum Parsimony method. For this alignment, the MEGA-6 (Molecular Evolutionary Genetics Analysis) software was used.

Nucleotide accession number: The16S rRNA gene sequences of the phosphate solubilizing strain ASN-2 were submitted in NCBI data base (National Center for Biotechnology Information).

Statistical analysis

Readings were taken as the mean ± standard deviation of the mean of three replicates calculated using Microsoft Excel XP 2007.

Results and discussion

Isolation of rhizobacteria from Piper betle rhizosphere samples

A total of fifteen rhizobacterial strains were isolated from the rhizosphere soil samples of *Piper betle* fields of Guntur district (Nutakki, Revendrapadu) and they were designated as ASN-1 to ASN-15.

Table 1. Phosphate solubilization index (SI) of the strain ASN-2

Strain	Diameter of zone (D) of clearance (mm)	Diameter of growth (d) of colony (mm)	D/d ratio
ASN-1	2	10	0.2
ASN-2	35	8.5	4.1
ASN-3	2	8	0.25
ASN-4	8	12	0.66
ASN-5	28	18	1.5
ASN-6	2	4	0.5
ASN-7	6	4	1.5
ASN-8	14	11	1.2
ASN-9	1	3	0.33
ASN-10	2	10	0.2
ASN-11	2	4	0.5
ASN-12	10	8	1.25
ASN-13	4	8	0.75
ASN-14	2	10	0.2
ASN-15	5	3	1.6

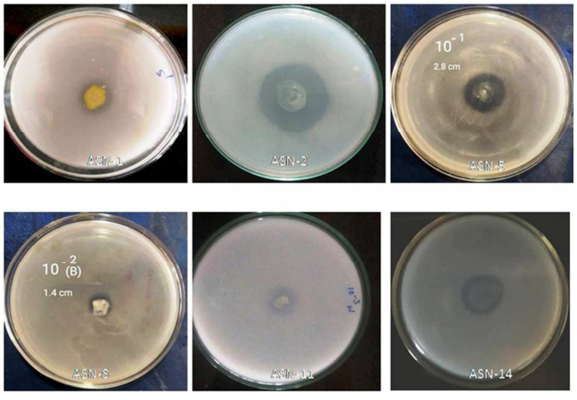

Fig.1. Formation of Phosphate solubilization of different isolates in Pikovskaya's agar medium

Screening of rhizobacterial strains for phosphate solubilization

All the 15 isolated bacterial strains were subjected to screening of their phosphate solubilizing ability by using Pikovskaya's agar medium (PKA) supplemented with 0.50% of tri-calcium phosphate. A loopful of 48h old-bacterial cultures were spot inoculated on PKA plates and growth was observed after 3 days (Sujatha and Ammani, 2014). Among the 15 strains tested, interestingly, the isolate ASN-2 exhibited significant zone of phosphate solubilization (28mm) (Table 1, Fig. 1).

Table 2. Biochemical and physiological characterization of the strain ASN-2

Characteristics	ASN-2
Gram's reaction	-
Cell morphology	Rod shaped
Fluorescence on King's B medium	+
Temperature for growth	28±2°C
pH for growth	6.8±2
Catalase activity	+
Oxidase activity	+
Citrate utilization test	+
Methyl red reaction	-
Indole production test	-
Voges-proskauer reaction	-
H_2S production	+
Starch hydrolysis	+
Nitrate reduction reaction	+

(+) indicates positive; (-) indicates negative

Estimation of phosphate solubilization efficiency

The phosphate solubilizing capacity of the strain ASN-2 strain in Pikovskaya's broth indicates that, the strain has efficiently solubilized the inorganic phosphate (tri-calcium phosphate). Maximum P-solubilization was detected after 96h of incubation period. ASN-2 strain was produced 197.32 µg/mL^{-1} soluble phosphate in the PK broth after incubation period and pH was decreased up to 5.0 from initial pH 7.0. Similarly the highest phosphate solubilization efficiency of *Bacillus* spp. was noticed after incubation of 96h (Banerjee *et al.*, 2010). Paul and Sinha (2017) reported the strain *Pseudomonas aeruginosa* KUPSB12 efficiently solubilized the inorganic phosphate and produced the 219.64 µg/mL^{-1} of soluble phosphate after 96 h of incubation period.

Biochemical and physiological characteristics of the strain ASN-2

The physiological and biochemical characteristics were main tools for identification of bacteria. Several tests were conducted for identifying strain ASN-2 (Table 2).

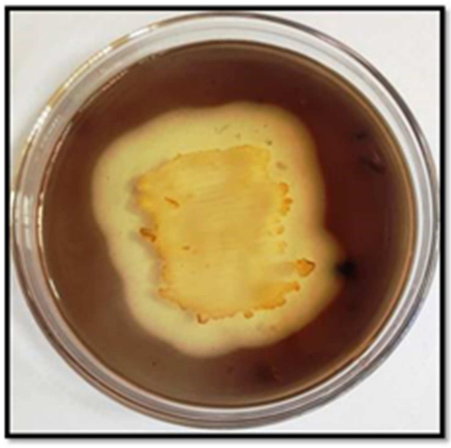

Fig. 2. Starch hydrolysis of the strain ASN-2

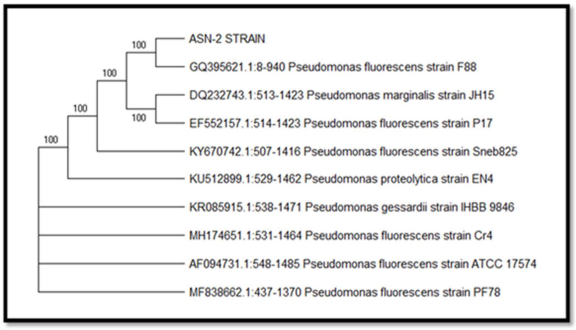

Fig. 3. Maximum Parsimony Tree based on partial 16s r RNA Gene Sequence showing relationship between strain ASN-2 and related members of the genus Pseudomonas

The strain shown negative for Gram's reaction and cells with rod shaped. The strain was cultured on King's B agar medium (KB) and exhibited significant fluorescent appearance under the UV light. The strain ASN-2 exhibited a positive response to catalase activity, oxidase activity and citrate utilization but negative to methyl red, indole production and Voges-Proskauer reactions. The strain ASN-2 possesses positive reaction to H_2S production, Nitrate reduction reaction and starch hydrolysis (Fig. 2).

Molecular identification of the strain ASN-2

The phylogenetic tree was constructed by using maximum parsimony method through bootstrap analysis and the strain ASN-2 was identified as *Pseudomonas fluorescens* (Fig. 3). The 16S r RNA sequences of the bacteria were submitted in NCBI Genbank database under the accession number is MW537707. The sequence was aligned and compared with the 16S r RNA gene sequences available in the Genbank database using the multi-sequence advanced BLAST comparison tool. The phylogenetic analysis of the 16S r RNA gene sequence was aligned by using MEGA-6 software.

Conclusion

Fluorescent *Pseudomonas* is commonly found in rhizosphere of different crops. Potential phosphate solubilizing *Pseudomonas fluorescens* ASN-2 isolated from the rhizospheric soil of *Betel vine* plant could be used as a potential phosphate solubilizer in agricultural environments.

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