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Phosphate-solubilizing microbes and their occurrence in the rhizospheres of *Piper betel* in Karnataka, India

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Abstract: Low phosphate solubility is one of the most important factors limiting the plant growth in Indian soils. Many microorganisms can enhance phosphate solubility, but little is known about the magnitude of their phosphorus-solubilizing ability. The native populations of phosphate-solubilizing bacteria and fungi were studied in different rhizospheric soil samples obtained from betel vine plants (*Piper betel* L.) in order to compare the results. The present study focuses on the phosphate-solubilizing capacity of bacteria and fungi in rhizospheric soil samples obtained from betel vine plants, revealing the dominance of *Aspergillus* species (26.1 mm) as major phosphate solubilizers, along with *Bacillus subtilis* (46.6 mm) among the bacteria that utilize tricalcium phosphate, potassium dihydrogen phosphate, and rock phosphate as phosphate sources. The other phosphorus-solubilizing microorganisms were *Bacillus* species, *Streptomyces*, *Aspergillus fumigatus*, *Nocardia*, actinomycetes, and certain yeasts. The presence of high numbers of phosphate-solubilizing bacterium *Bacillus subtilis* (3×10^6 cfu g^{-1}) and fungus *Aspergillus niger* (3×10^5 cfu g^{-1}) in the rhizospheric zones of *Piper betel* plants explains how the plants obtain their nutrient requirements. The identity of *Aspergillus* species and *Bacillus* with the maximum zone was confirmed using molecular sequencing with 16s rDNA. The sequence data were aligned and analyzed to identify the bacteria along with their closest neighbors.

Key words: *Aspergillus*, *Bacillus*, phosphates, *Piper betel*, rhizosphere

Introduction

The betel vine plant (*Piper betel* L.) is a climber with spicy, heart-shaped leaves that are consumed by about 20 million people across Asia. It is cultivated widely in southeastern Asian countries and used variously for both traditional and medicinal practices. The rhizospheric soil of the betel vine plant has shown a varied group of microorganisms, some of which have potent phosphate-solubilizing capacities.

Phosphorus is one of the most limiting factors in crop production in many kinds of soils in different geographical regions as a result of high phosphorus fixation. A large amount of soluble inorganic phosphate applied to the soil in the form of chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to the plants (1). The concentration of soluble phosphorus (P) in many soils is usually very low compared to other mineral nutrients, which are present in a range of millimolar amounts; phosphorus is only available in micromolar quantities or less. The majority of the phosphorus that is applied to the soil is fixed rapidly into forms that are poorly available to the plant roots. Most of the inorganic phosphates in acidic soils are salts from iron or aluminum, whereas calcium phosphates are the predominant forms in neutral or calcareous soils (2-4).

Phosphorus is a plant macronutrient that plays a significant role in plant metabolism, ultimately reflected on crop yields. It is important for the functioning of key enzymes that regulate the metabolic pathways (5). It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorus, which is necessary to support maximum plant growth. The uptake of phosphorus by the plant is only a small fraction of what is actually added as phosphate fertilizer (6). The remaining phosphorus is later converted to insoluble forms of phosphates and lost in the soil due to adsorption, precipitation, or conversion to organic phosphates (7). Phosphorus deficiency is widespread and phosphorus fertilizers are required to maintain crop production. When it is added to the soil in the form of phosphatic fertilizer, only a small portion is utilized by plants; the rest is converted into insoluble phosphates (8).

Soil microorganisms play an important role in making the phosphorus available to plants by mineralizing the organic phosphorus in the soil. These microorganisms have been isolated from a number of different soils in India (9). 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. These bacteria and fungi have the potential to be used as biofertilizers. Their role in increasing the soil nutrient value is of utmost importance. Their application to crop fields has resulted in an increased yield of several crops, such as cereals, legumes, fibers, vegetables, oils, and other crop plants (10).

PSMs have been well studied and their isolation and role in crop production have been noted. Studies on the distribution of these organisms have been conducted in several soils. The information available is insufficient for the study of the diversity of these organisms; many of these organisms exhibit stress tolerance and can adapt themselves to varying environments (11). Rhizospheric phosphate-solubilizing bacteria and fungi 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. They can be divided into 2 groups: phosphate-solubilizing bacteria (PSB) and phosphate-solubilizing fungi (PSF). Fungal diversity affects soil agglomeration, thereby increasing the soil quality and fertility; the health of the plant is thus affected directly. Some bacterial and fungal organisms have solubilizing potential for organic and inorganic phosphorus, respectively (12). Phosphorus-solubilizing activity is determined by the ability of microbes to release metabolites such as organic acids, which, through their hydroxyl and carboxyl groups, chelate the cation bound to the phosphate, the latter being converted into soluble forms. Phosphate solubilization takes place through various microbial processes or mechanisms, including organic acid production and proton extrusion (13). PSMs dissolve the soil P through the production of low-molecular-weight organic acids, mainly gluconic and ketogluconic acids (14), in addition to lowering the pH of the rhizosphere (15).

It is thus essential that wider study be done to explore the natural biodiversity of soil organisms and their optimization. In addition, researchers must continue to examine microbial interactions for use as biofertilizers, biopesticides, and growth promoters; microbial communities in field soils must also be developed (10).

Many studies have demonstrated that soil-borne microbes interact with plant roots and soil constituents at the root-soil interface (16).

The principal aim of this investigation was to isolate the phosphate-metabolizing microorganisms (bacteria and fungi) from the rhizospheric soils of betel vine plants grown in Karnataka, India. The study further aimed to detect the phosphate-solubilizing efficiency of rhizospheric bacteria and fungi with 3 different sources of phosphates: tricalcium phosphate (TCP), potassium dihydrogen phosphate (KHP), and rock phosphate (RP). Additionally, we conducted a comparative analysis of the solubility of inorganic phosphates by the rhizospheric PSMs and identified the predominant microorganisms at the molecular level.

Materials and methods

Soil analysis

Betel vine plant (*Piper betel* L.; Piperaceae) rhizosphere soil samples were collected from 4 different betel cultivating areas: Murunadu, Beerur (with samples from 2 different Beerur locations), and Bangalore regions of Karnataka, India. Soil samples were collected from 10 randomized young and old betel vine plant rhizospheres in order to obtain as much possible variability in the microorganisms for a comparative analysis. To maintain uniformity, the soils were taken from within 20-cm vicinities of the plants and from depths of 5-10 cm from the surface during the months of October and November. The soil samples were dried, crushed, and passed through a 2-mm sieve before being mixed into a single composite sample. These soil samples were then analyzed and the characteristics of the soils were tabulated.

Isolation of rhizospheric microflora

From each soil sample, 1 g of soil was suspended in a 9-mL blank and serially diluted. The dilutions were plated on Pikovskaya's (PVK) medium in order to isolate the PSB and PSF (17-19). Those colonies surrounded with a halo zone were transferred to PVK medium 3 times in order to maintain the purity of the culture.

Isolation of PSMs

Phosphate solubilization on PVK medium was examined by growing the different isolates on PVK medium substituted with TCP, KHP, and RP. The concentrations of TCP, KHP, and RP used in the media were varied by taking 2.5 g L^{-1}, 5.0 g L^{-1}, and 7.5 g L^{-1} in the basal PVK medium (20-22). The plates were inoculated using point inoculation and incubated at 28 °C for 3 days and 5 days for bacteria and fungi, respectively. The solubility of phosphate was observed as a zone of clearance with a diameter that was measured in millimeters and taken in triplicate (9). The microbial phosphorus solubilization trait was analyzed by determining the P-solubilization efficiency (PSE).

PSE = diameter of entire colony/diameter of clearing zone × 100

The efficient P-solubilizing bacterial species were then further identified (23-25).

Identification of PSMs

The identification of bacterial species was done using pure cultures obtained from the various soil isolates (9). The bacterial culture was identified to its nearest species based on 16s rDNA sequence data. Genomic DNA was isolated from the pure culture of *Bacillus* sp. and an rDNA fragment of approximately 1.4 kb was amplified using high-fidelity polymerase chain reaction (PCR) polymerase. The PCR product was sequenced bidirectionally using the forward, reverse, and internal primers. The sequence data were aligned and analyzed to identify the bacteria and their closest neighbors.

PCR amplification conditions were 1 µL of DNA taken and amplified using 400 ng of 16s rDNA forward primer 5'–AGAGTRTGATCMTYGCTWAC-3', 400 ng of reverse primer 5'-CGYTAMCTTWTTACGRCT-3', and Taq polymerase enzyme (4). The phylogenetic tree builder used sequences aligned with alignment software. A distance matrix was generated using the Jukes-Cantor corrected distance model. While generating the distance matrix, only alignment inserts were used, and the minimum comparable position was 200. The tree was created using Weighbor with alphabet size 4 and length size 1000 (26).

Phylogenetic dendrograms were constructed using a phylogenetic tree builder with sequences aligned with alignment software (bootstrap method). The approach was to create a pseudoalignment by taking random positions of the original alignment. The pseudoalignment was as long as the original alignment and was used to create a distance matrix and a tree. The process was repeated 100 times and a majority consensus tree was obtained (27).

The fungi were identified by extracting the DNA from the pure culture. The ITS region of the rDNA was amplified by universal primers ITS 4 and 5 and subsequently sequenced. The crude sequence was manually edited and aligned with those available in the National Center for Biotechnology Information (NCBI) database (27).

Experimental design and statistical analysis

Three replicates were used for the isolation of bacteria and fungi from the rhizosphere of betel vine plants. Viable counts and a determination of the PSE

of the PSMs were made for each species in triplicate. A replicate consisted of one plate of PVK medium with a single organism inoculated in the center. From each replicate, 3 readings of the zone of inhibition were recorded in millimeters. Results are presented as the mean zone of inhibition for each organism. Molecular identification of the most efficient PSB and PSF was done, and the sequence of bases for *Bacillus subtilis* and *Aspergillus niger* are presented.

The data obtained were statistically analyzed for analysis of variance (ANOVA) for significance at $P \leq 0.05$ with the ORIGIN software program (28).

Results and discussion

Soil analysis

The physicochemical analysis of the soil indicated a low concentration of phosphates in the soil samples tested. Specifically, 2 soil samples, Beerur sample 1 and the Bangalore sample, showed low concentrations of phosphates at pH 7.1 and the presence of high levels of organic carbon (Table 1). The second soil sample from Beerur, with a pH of 7.3, had higher organic carbon content and higher phosphorus, which was shown to limit other nutrients such as potash, zinc, copper, and manganese. In these areas in the Western Ghats region of India, the betel plant is cocultivated with several trees, including coconut, areca nut, and coffee (29). Betel vine plants are grown extensively in areas that receive heavy rainfall combined with sloping terrain; this combination results in a loss of nutrients in the soil and causes low productivity and phosphorus content.

Rhizospheric microflora

Bacteria

Several bacterial and fungal forms were isolated from the rhizospheric soil samples of *P. betel*. The predominant bacterial flora isolated showed the presence of *Bacillus*, *Arthrobacter*, diphtheroids, and actinomycetes (Table 2). The bacterial organisms were identified according to staining and cultural characteristics. The dominant microorganisms were cultured in pure cultures on slants maintained for further testing.

Fungi

The fungal isolates obtained from the various *P. betel* rhizosphere soils were identified as species of *Aspergillus*, *Fusarium*, *Penicillium*, and *Verticillium* (Table 3).

PSMs

The 4 rhizospheric soils of *P. betel* showed the presence of several bacterial and fungal species that were able to solubilize phosphates. The primary isolation on PVK medium with TCP clearly

Table 1. Soil characteristics of the rhizospheric soil of *Piper betel*.

No.	Soil sample	Geographic location	Soil type	pH	Organic carbon %	Phosphate (kg/ha)
1	Murunadu sample	Murunadu, Karnataka, India 12°18′N, 75°45′E	Black, clayey, high moisture, soft soil	7.7	2.5	42
2	Beerur sample 1	Beerur, Karnataka, India 13°53′N, 75°58′E	Red loamy soil, dry, coarse granules	7.1	1.15	7.41
3	Beerur sample 2	Beerur, Karnataka, India 13°53′N, 75°58′E	Red loamy soil	7.3	0.90	69.19
4	Bangalore sample	Bangalore, Karnataka, India 12°58′N, 77°48′E	Red loamy clay soil	8.3	0.85	7.41

Table 2. Phosphate-solubilizing bacteria isolated from the *Piper betel* rhizosphere.

No.	Soil sample	Organisms isolated	Colony count (colony forming units in cfu g^{-1})	Percentage of organism	Average zone of clearance of phosphate (mm)
1	Murunadu sample	*Bacillus* sp. 1	2×10^3	0.06	4
		Diphtheroids	7×10^4	2.33	0
		Arthrobacter	4×10^4	1.33	4
2	Beerur sample 1	*Bacillus* sp. 2	3×10^3	0.10	4
		Actinomyces	4×10^4	1.33	9
3	Beerur sample 2	*Bacillus*	3×10^4	1.0	5
		Bacillus	2×10^5	6.66	8
		Bacillus	3×10^6	100	9
4	Bangalore sample	*Bacillus*	4×10^4	1.33	9
		B. subtilis	3×10^6	100	10

Table 3. Phosphate-solubilizing fungi isolated from the *Piper betel* rhizosphere.

No.	Soil Sample	Organisms isolated	Colony count (colony forming units in cfu g^{-1})	Percentage of organism	Average zone of clearance of phosphate (in mm)
1	Murunadu sample	*A. fumigatus*	2×10^5	50.0	8
		A. flavus	3×10^3	0.75	10
		Yeast	2×10^4	5.00	8
2	Beerur sample 1	*A. niger*	3×10^5	75.0	12
3	Beerur sample 2	*A fumigatus*	4×10^5	100	9
4	Bangalore sample	*A. flavus*	3×10^4	7.50	8
		A. clavatus	2×10^4	5.00	2

showed the activity of several bacterial and fungal forms, including *Bacillus*, *Streptomyces*, yeast, and *Aspergillus*, as phosphate solubilizers. The PSMs with the most phosphate-solubilizing ability were *Bacillus* among the bacterial and *Aspergillus* among the fungal isolates. The results indicated greater numbers of microorganisms showing the maximum zone of clearance of phosphates on PVK medium.

The PSM count was around 10 to 100 times lower than the bacterial count in the rhizospheric samples. The count of *Bacillus* sp. in the rhizospheric soils was 3×10^4 cfu g^{-1}, while the PSB count was in the range of 1×10^3 cfu g^{-1}. Similarly, the counts of the PSF were much lower than the counts of the rhizospheric fungi (Tables 2 and 3). The PSF count ranged from 2×10^3 cfu g^{-1} to 4×10^3 cfu g^{-1} for *Aspergillus* sp. (30). The Murunadu soil sample showed 3 different types of PSF: *A. fumigatus*, *A. flavus*, and yeast isolates varying from 2×10^3 cfu g^{-1} to 2×10^6 cfu g^{-1}.

Phosphate solubilization

The screening of microbial isolates for phosphate solubilization revealed variations among the different groups of bacteria and fungi. In our study, 6 isolates of bacteria and 6 isolates of fungi were found to solubilize different phosphates to varying degrees (Tables 4 and 5).

Table 4. Phosphate solubilization of different phosphates by bacterial isolates.

Organism	TCP (g L^{-1}) solubilization zone of inhibition (mm)			KHP (g L^{-1}) solubilization zone of inhibition (mm)		
	TCP2.5	TCP5.0	TCP7.5	KHP2.5	KHP5.0	KHP7.5
Streptomyces sp.	28.5 c	7.5 b	9 a	4.1 b	5.1 b	3.6 c
Bacillus sp.	46.6 b	4.5 c	5.1b	4.3 b	6.7 b	13a
Actinomyces sp.	8.8 d	1.0d	--	8.3 a	9.2 a	7.8 b
Yeast isolate	30.5 c	6.6 b	4.2b	2.3 c	5.7 b	4.6 c
Bacillus subtilis	82.6 a	12.8 a	2.4c	4 b	3.4c	2.4 d

Values with different letters are significant at $P < 0.05$.

Table 5. Phosphate solubilization of different phosphates by fungal isolates.

Organism	TCP (g L^{-1}) solubilization zone of inhibition (mm)			KHP (g L^{-1}) solubilization zone of inhibition (mm)			RP (g L^{-1}) solubilization zone of inhibition (mm)		
	TCP2.5	TCP5.0	TCP7.5	KHP2.5	KHP5.0	KHP7.5	RP2.5	RP5.0	rp7.5
A. flavus str. 1	21.5 b	0	0	20.5 a	11.6 b	13.4 a	29.3 a	7.8 c	21.2a
A. terreus	26.1 b	5.8 c	0	5.8 b	7.4 c	6.7 b	8.8 b	7.7 c	5.8b
A. clavatus	27 b	5.6 c	7.8 a	3.6 c	4 c	3.7 c	7.7 b	7.3 c	4.4b
A. flavus.str. 2	30.8 a	5 c	6.6 b	5.3 b	6.6 c	3.5 c	6.5 c	7.7 c	1.1d
A. fumigatus	10.2 c	8 b	2.7 c	2.4 c	5.7 c	6.3 b	32.6 a	11.2 a	25.5a
A. niger	34.4 a	17.5 a	0	24.8 a	18.1 a	13.7 a	9.4 b	8.3 b	3.1c

Means with different letters are significant at $P < 0.05$.

TCP

Bacteria

TCP was readily solubilized by bacteria and fungi at a concentration of 2.5 g L^{-1}. The predominant bacterium utilizing TCP at 2.5 g L^{-1} was *Bacillus subtilis* (82.6 mm), which showed the highest zone, followed by *Bacillus* sp. (46.6 mm). The actinomycetes solubilized the phosphates at a very low level, as indicated by a zone of 8.8 mm. When the concentration of TCP was increased to 5.0 g L^{-1}, *Bacillus subtilis* continued to show a predominance, with a zone of 12.8 mm (Table 4).

Fungi

Among the fungal isolates, the maximum zone was obtained with *Aspergillus niger* at a concentration of 2.5 g L^{-1} (34.4 mm), followed by *A. flavus* str. 2 (30.8 mm). *A. niger* (17.5 mm) solubilized TCP better at a concentration of 5.0 g L^{-1}, whereas *A. flavus* str. 1 did not solubilize TCP at any of the tested concentrations (Table 5).

KHP

Bacteria

The bacterial isolates that most effectively solubilized KHP at all concentrations of the

phosphates were the actinomycetes, providing zones of 8.3 mm, 9.2 mm, and 7.8 mm for concentrations of 2.5 g L^{-1}, 5.0 g L^{-1}, and 7.5 g L^{-1}, respectively.

Fungi

The solubilization of KHP by bacteria and fungi varied at different concentrations. KHP was efficiently solubilized at 2.5 g L^{-1} by *A. flavus* str. 1 (20.5 mm) and *A. niger* (24.8 mm). The overall solubilization was greatest for *A. flavus* str. 1 (11.6 mm) and *A. niger* (18.1 mm) for KHP at 5.0 g L^{-1}. KHP was solubilized best at 7.5 g L^{-1} by *A. niger* (13.7 mm).

RP

Bacteria

RP failed to be solubilized by the bacterial isolates at all concentrations.

Fungi

RP was well solubilized by the fungal isolates at 2.5 g L^{-1}, and at 5.0 g L^{-1} by *A. flavus* str. 1 (29.3 mm and 7.8 mm) and *A. fumigatus* (32.6 mm and 11.2 mm).

Among the different PSB, the major phosphate solubilizer, *Bacillus*, was identified to its nearest species based on 16s rDNA sequence data.

Molecular identification of *B. subtilis*

Analysis of the rDNA fragment (approximately 1.4 kb) of the bacterium *B. subtilis* was done using high-fidelity PCR polymerase amplification. The DNA ladder used for PCR amplification was a 500-bp ladder containing 10 DNA fragments and 1 kb, respectively (LAD 02 and LAD 03). The sequence data obtained after the amplification of the DNA (Table 6) showed the molecular identification of the bacterium to be *B. subtilis* subsp. *subtilis*; RB14; FJ263381. The closest neighbor homolog was found to be *Bacillus amylofaciens*; 7-70; fj378040. The hierarchy of the organism suggests its identification. The alignment view and distance matrix for *B. subtilis* showed a 0.961 S.ab. score with *B. subtilis* subsp. *subtilis* 3EC2A10 of NCBI Acc. No. EU304917 (4,27). The phylogenetic tree was generated using the Jukes-Cantor distance model (Figure) (26).

Table 6. The aligned sequence data obtained for *Bacillus subtilis* subsp. *subtilis* isolated from Bangalore soil.

>GP
GGGGAAGTGGTGGCTTGCTCATGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCG
GATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAA
AGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGT
TTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTT
AGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGAC
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATC
CTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAACCGCGAGGTTA
AGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC
GTGAAAGCTGGAATCGCTAATAAACGCGGAACAGCATATGCGCGTCTTTT

Table 7. The aligned sequence data obtained for *Aspergillus niger* (approximately 554 bp) isolated from Beerur sample 1.

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GCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCG
GCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGC
GTGCGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA
GAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTG
CGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGG
TCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGG
GGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTTTTTCCAGGTTGACCT
CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA
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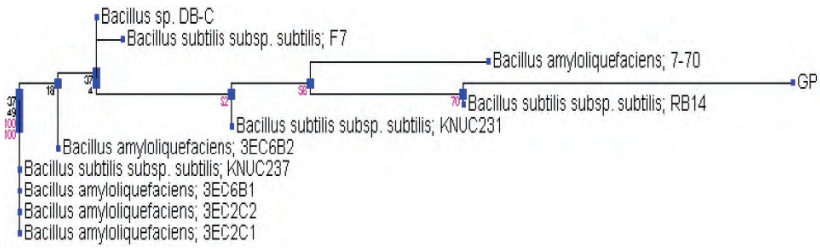

Figure. The phylogenetic tree of *B. subtilis* subsp. *subtilis* isolated from Bangalore soil with optimum phosphate-solubilizing capacity.

Fungal identification

The 6 strains of *Aspergillus* used in the study of phosphate solubilization were identified based on morphological and cultural features. They were identified as *A. flavus* str. 1, *A. terreus*, *A. clavatus*, *A. flavus* str. 2, *A. fumigatus*, and *A. niger*.

The strain of *A. niger*, which showed a good zone of clearance with different phosphates, was identified using genomic DNA extract from the pure culture. It possessed 100% ITS sequence similarity with the 7 *Aspergillus* species given below (Table 7).

1. *Aspergillus vadensis* Samson RP de Vries & Frisvad (2005)

2. *A. tubingensis* (Mosseray) Kozak. (1934)

3. *A. foetidus* Thom & Raper (1945)

4. *A. costaricaensis* Samson & Frisvad (2004)

5. *A. niger* var. *niger* Tiegh. (1867)

6. *A. awamori* Nakaz. (1915)

7. *A. niger* var. *phoensis* (Corda) Al-Musallam (1980)

Many of the species isolated constitute the indigenous flora of Western Ghats, Karnataka, India. The predominant species of *Aspergillus* were identified to be *A. flavus*, *A. niger*, *A. ochraceous*, *A. terreus*, and *A. fumigatus* (21).

In the present study, soils with higher organic carbon content showed a greater number and variety of PSB and PSF. Although no reports were available on the exact relationship between the total bacterial and fungal populations and that of the PSB, it is possible that the organic carbon in the soil enhanced the growth of both rhizospheric organisms and phosphate-solubilizing organisms, as reported by Venkateshwarulu et al. (31) and Gupta et al. (32).

The results of phosphate solubilization were compared with studies on PSB from the rhizospheric soils of chickpeas (10). The occurrence of PSMs in the rhizospheric soil samples of various crop plants has been reported earlier by various researchers (9,33-35).

PSMs are an ecologically special functioning group of soil microorganisms that play an important

role in the turnover of organic phosphate and insoluble inorganic phosphate and in the cycling of phosphorus in the soil (35).

The present investigations indicate the presence of phosphate-solubilizing organisms, especially species of *Aspergillus* and *Bacillus*, capable of solubilizing different sources of phosphates, including TCP, KHP, and RP. It is well known that PSMs in the soil solubilize insoluble phosphates, mainly by secreting acids into the medium (34,36). The solubilization of TCP, KHP, and RP varied with the different fungal and bacterial organisms used.

The results clearly indicate that TCP and RP were solubilized most easily by bacteria and fungi at a concentration of 2.5 g L^{-1}. The solubility of KHP varied at different concentrations. The findings of the phosphate solubilization of *A. niger* for the 3 different phosphate sources, TCP (34.4.1 mm), KHP (24.8 mm), and RP (9.4 mm), clearly indicated an optimum, efficient solubilization for TCP (10,37). However, good solubilization of TCP was also shown by *A. niger* (34.4 mm) and *A. flavus* str. 2 (30.8 mm). The strain showing the maximum zone was *Bacillus subtilis* (82.6 mm), with TCP at a concentration of 2.5 g L^{-1}, followed by *B.* sp. (46.6 mm) for other concentrations of TCP.

The results for phosphate solubilization were compared to those obtained for different bacterial and fungal isolates tested against different phosphate sources, TCP and RP, from other similar experiments (21,24,37,38). Comparing the phosphate solubility of the 3 phosphates in the present study, it is evident that a greater degree of solubility was shown by *A. niger* at lower concentrations, while the maximum degree of solubility was shown by *A. terreus* for TCP at a concentration of 2.5 g L^{-1}. An increased concentration of TCP (7.5 g L^{-1}) resulted in a decrease in the phosphate solubility levels of the tested isolates. In the case of the bacterial isolates, one of the predominant isolates was found to be *B. subtilis*. The optimum concentration for TCP solubilization by bacteria and fungi was seen at 5.0 g L^{-1}. The results varied significantly among the various bacterial and fungal isolates, as shown by ANOVA.

All fungi tested were able to solubilize TCP, KHP, and RP in a solid culture state. The degree of solubility of phosphates varied with the different species of *Aspergillus* tested. The fungal isolate *A. niger* showed a maximum zone of clearance at 17.5 mm for TCP at a concentration of 5.0 g L^{-1}. The smallest zone of clearance was shown by isolate *A. flavus* str. 1 (0 mm at a TCP concentration of 5.0 g L^{-1}) (37-39).

The bacterial isolates failed to demonstrate the ability to solubilize RP. However, varied results were obtained with the fungal isolates. *A. fumigatus* showed high solubilization with RP, with zones of 25.5 mm (7.5 g L^{-1}), 11.2 mm (5.0 g L^{-1}), and 32.6 mm (2.5 g L^{-1}). Conversely, *A. flavus* str. 1 showed the least solubility with a RP concentration of 2.5 g L^{-1}.

PSMs, acting in unison with plant roots, are responsible for the solubilizing of phosphatic minerals in the soil. They interact in the microenvironments of the rhizosphere, converting a portion of the insoluble phosphates into soluble forms (40).

The finding that several species of *Aspergillus* are RP solubilizers is of great importance, since RP has applications as a fertilizer and can be directly applied to the soil. *A. niger* and *A. flavus* have been studied as RP solubilizers by several investigators, whose studies are in accordance with our findings (19,39,40). Several varieties of PSMs solubilize RP and make it available to plants; previous research has also been conducted on the solubilization of RP in liquid cultures by *A. niger* (41-43).

The molecular characterization for *A. niger* gave the base similarity sequence for *A. niger* (Table 5), which was found to be nearest to *A. awamori* Nakaz. (1915) and *A. tubingensis* (Mosseray) Kozak. (1934). The present study indicates that these PSMs were abundant in the soils of regions where betel vine plants were cultivated. These phosphate solubilizers play an important role in the conversion of insoluble phosphates to the soluble forms of phosphates, thereby making them available to plants. A large number and diverse variety of PSMs are seen in the rhizosphere of *P. betel* plants. The dominant phosphate solubilizers were *B. subtilis* subsp. *subtilis*; RB14; FJ263381 and *Aspergillus niger* and *A. awamori*. Great genetic diversity exists among PSB (22).

The results in the present investigation point to the presence of a diverse group of PSMs inhabiting

the rhizosphere of betel vine plants in the different growing regions of Karnataka, India. It is evident that PSMs are widely distributed, and significant variations were noted among the microbes with respect to their PSE. The use of TCP, KHP, and RP along with the phosphate solubilizers *A. niger* and *B. subtilis* subsp. *subtilis* as phosphatic fertilizers could enhance the phosphate solubility of the soils. Further field studies are required to confirm the application of these organisms at the field level.

These microorganisms can be exploited and used as potential phosphatic biofertilizers for the cultivation and growth of *P. betel*, a plant whose leaves are nutritive and contain anticarcinogens showing promise for the manufacturing of blood cancer drugs. The essential oils obtained from *P. betel* leaves are used in manufacturing medicines, perfumes, mouth fresheners, and food additives.

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