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PHOSPHATE SOLUBILIZING PLANT GROWTH PROMOTING MICROBES

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ABSTRACT

Plant growth promoting microbes dwelling in the rhizosphere are beneficial for plants. These microbes exhibit the capabilities to release plant growth regulators, hydrogen cyanide, siderophores, nodulation, nitrogen fixation, nutrient uptake, biocontrol and bioremediation. The ability of these microbes to increase phosphorus availability to the plants from soil plays a promising role in improving plant nutrition. Large quantity of phosphate present in soil or supplied in the form of chemical fertilizers becomes immobile via precipitation. The ability of phosphate solubilizing microbes to convert insoluble form of phosphorous into soluble form is an important attribute in sustainable farming for increasing crop yield. This ability of PSMs owes either to the production of organic acids or by the mineralization of organic phosphates by secreted acid phosphatases. This article gives an overview of plant growth promoting microbes in general and phosphate solubilizing microbes in particular with an emphasis on their isolation, characterization, biodiversity analysis, plant growth promoting ability and applicability of myconanoparticles as supplements and eventual substituents of chemical fertilizers.

Key words: Acid phosphatase, Biodiversity, Biofertilizers, Nanoparticles, Plant growth promotion, 16S rRNA, 18S rRNA

Introduction

The economic stability of an agricultural country like India is dependent on the crop yield. In order to meet the demands of growing population from gradually depleting and degrading land resources, the development of strategies for enhanced agricultural production is a great challenge for agricultural scientists. In the past, extensive efforts have been focused on increasing the efficiency of crop production with the application of chemical fertilizers. Although their application attributes to a boost in agricultural yield, their use is also associated with health hazards, environmental pollution and increased crop production cost [1].

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The use of plant growth promoting microorganisms (PGPMs) for example, *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Klebsiella*, *Fusarium*, *Aspergillus*, *Trichoderma*, *Helminthosporium*, *Phoma*, *Alternaria* sp. etc., is considered as an environment-friendly biotechnological alternative or a supplement to reducing the use of chemicals [2, 3].

Plant growth promoting microbes

Soil microorganisms inhabiting rhizosphere with beneficial effects on plant growth and health may be employed as a alternative to conventional agriculture [4]. These microbes are generally defined as plant growth promoting microbes (PGPMs). They are mainly defined by three inherent characteristics, viz., ability of colonizing roots, surviving and multiplying in microhabitats associated with the root surface in competition with other microbiota and promoting plant growth [5]. PGPMs inhabiting the rhizosphere exhibit beneficial effects on plant development by various direct and indirect mechanisms, for example, by suppressing the activity of plant pathogens through biological control, by producing hormone

(e.g., auxins and cytokinins), by releasing siderophores, by nodulation, nitrogen fixation, bioremediation and nutrient (e.g., phosphorus, nitrogen, etc.) uptake [6-10]. These mechanisms are active either simultaneously or sequentially at different stages of plant growth. On the basis of the effects exerted, PGPMs are usually categorized into two groups, those that enhance plant growth directly or those that enhance plant growth through their biocontrol activity (biocontrol PGPMs). The current paper is focused on PGPMs facilitating nutrient (phosphorus) uptake by plants. The mineral uptake is directly improved due to upsurges in specific ion fluxes at the surface of roots in the presence of PGPMs [11].

Phosphate solubilizing microbes

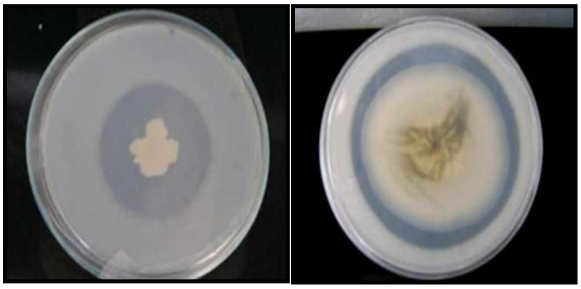
Phosphorus is one of the most essential elements in crop production playing an important role in various physiological activities including photosynthesis, cell division, good rooting system and utilization of carbohydrates. The plants take up phosphorus from soil, where it is available in the form of organic and inorganic phosphates. Most of the organic phosphoric compounds are orthophosphoric acid esters, including inositol phosphates, phospholipids and nucleic acids. Most inorganic phosphorous compounds in soil are calcium, iron or aluminum phosphates. In order to fulfill the nutritional requirements of plants, phosphorous is usually added to soils in the form of fertilizers, which are synthesized through high chemical- and energy-intensive processes [12]. However, plants are able to use only a small amount of this phosphorus as it is rapidly precipitated and fixed in soil by metal-cation complexes. To overcome such problem, solubilization and mineralization of phosphates by phosphate solubilizing microbes (PSMs) is one of the most important microbial physiological traits for plant growth promotion. Soil phosphate precipitated in the form of orthophosphate is likely to become bioavailable to plants through PSMs. The assimilation of soil phosphate by plants and microorganisms involves the production of acid phosphatase enzyme and organic acids with a consequent release of inorganic phosphorus (Pi) [13, 14]. PSMs thus play some part in correcting phosphorous deficiency within plants. Most of the PSMs reported so far include soil fungi, bacteria and yeasts. In general, among the whole microbial population within soil, phosphate solubilizing bacteria (PSBs) out-number phosphate solubilizing fungi (PSFs) constituting 1-50% and 0.1-0.5% of their total respective populations [15]. Although several PSBs occur in soil, their number do not outcompete other commonly established rhizospheric bacteria. As a result, the amount of inorganic phosphorus liberated by their action is generally not sufficient for significant plant growth promotion. Therefore, inoculation of plants with target PSMs at a concentration much higher than that normally present in soil is necessary to take optimal advantage of their phosphate solubilization ability for simultaneously enhancing plant yield and decreasing environmental pollution.

Characterization and genetic variability analysis of phosphate solubilizing microbes

Owing to the potential advantages of PSMs in plant growth promotion, the research on isolation and characterization of diverse microbes with higher phosphate solubilizing potential (PSMs) and analysis of interactions between microbes and their natural environment needs to be accelerated greatly. An integrated approach of morphological, biochemical and molecular markers is employed to identify superior strains of PSMs. As little is known about the diversity of phosphate solubilizers, the foremost impetus of the research is on the development of PCR based techniques to identify microbial community structure within soil. This involves rRNA-based analysis to explore microbial diversity and also to identify new strains. Metagenomic approaches involving isolation of total DNA from soil and amplification of microbial genes (bacterial 16S rRNA and fungal 18S rRNA genes) using gene-specific PCR primers are now being employed.

At our end, in an attempt to isolate and characterize PSMs, twenty one bacterial and twenty fungal isolates with phosphate solubilizing potential were isolated on the basis of plate assay on Pikovskaya's medium [16] from different regions of Lucknow, wherein positive isolates showed clear halo zones around the colonies on medium containing tricalcium phosphate. One positive bacterial and one positive fungal isolate showing clear halo zones are shown in Figure 1. Phosphorus solubilization ability of PSMs has direct correlation with pH, because PSMs dissolve the soil phosphate through production of low molecular weight organic acids, namely gluconic and ketogluconic acids, consequently lowering the pH of rhizosphere [17]. The lowering of rhizospheric pH is associated with the biotical production of proton/ bicarbonate (anion/ cation balance) and gaseous (O_2/

CO_2) exchanges. A decrease in pH owing to the production of organic acids was observed in each case, which was analyzed on plate by bromophenol blue staining, wherein yellow coloration depicted lowering of pH.



(a) **(b)**

Figure 1: **Selective screening of phosphate solubilizing bacteria (a) and fungi (b) by plate assay procedure showing clear halo zones**

The bacterial isolates were subjected to morphological, biochemical and molecular characterization. The colony characteristics of bacterial isolates were determined with respect to colony appearance, form, texture and Gram staining property. The biochemical characterization of positive bacterial isolates was based on the measurement of solubilization index (SI = Colony diameter + halo zone diameter / Colony diameter) on Pikovskaya's medium [16], lowering of pH of the medium and phosphatase enzyme activity [18]. Further characterization of isolates with high phosphate solubilizing ability was also done by various routine tests including production of oxidase, catalase, urease, nitrate reductase, amylase, citritase and H_2S using standard protocols. The sequence of the 16S rRNA gene has been widely used as phylogenetic marker in microbial ecology since the extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species [19]. Thus, six bacterial isolates showing maximum solubilization index, maximum reduction in pH and the highest acid phosphatase enzyme activity were subjected to identification at molecular level by amplification of 16S rRNA gene portions from different bacterial samples using forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer: 5'-GTTACCTTGTTACGACTT. The PCR products were sequenced by Amnion Biosciences Pvt. Ltd. 16S rRNA sequence was analyzed with the nucleotide database available at the GenBank using the BLAST search at NCBI for identification of bacteria. Sequences were aligned using ClustalX version1.81 and phylogenetic tree were prepared by MEGA3 software and analyzed. DNA accession numbers of each strain were obtained from DNA Data Bank. The results are tabulated in Table 1.

The fungal isolates were identified by the appearance of colonies on the Sabouraud agar medium and microscopic examination of mycelial and spore characteristics using standard cover-slip technique and lactophenol cotton blue staining procedure. The biochemical characterization of positive fungal isolates was based on the measurement of solubilization index, lowering of pH of the medium, phosphatase enzyme activity and various routine tests including production of amylase and utilization of citrate. The internal transcribed spacer (ITS) region of rRNA genes (rDNA) is the most widely sequenced DNA region in fungi and it exhibits a wide degree of variations as compared to other genic regions of rDNA amongst various species [20]. Thus, ITS regions have typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Thus, for characterization of isolated fungal species at molecular level, the amplification of 18S rRNA gene portions from different fungal samples was carried out using forward primer (ITS 5): 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primer (ITS 4): 5'-TCCTCCGCTTATTGATATGC-3' [21]. PCR products were sequenced as described for bacterial isolates. The results are given in Table 2.

RAPD (Randomly amplified polymorphic DNA) analysis, which employs short primers of arbitrary sequences to amplify random portions of the sample DNA by PCR, is used to compare a number of microbial communities and quantify their overall similarity [22]. Thus, in order to determine genetic diversity, RAPD analysis of the six bacterial and six fungal isolates exhibiting high phosphate solubilizing potential was performed using twenty different OPA (Operon Technologies, Alameda, USA) random decameric primers. The NTSYS 2.02 software package [23] was used for generation of a dendrogram of genetic relationship. The results showed remarkable diversity amongst the isolates (data not shown).

Plant growth promoting ability of phosphate solubilizing bacteria

Healthy soil contains plenty of beneficial microorganisms, which play a significant role in providing mineral nutrition to growing plants.

Table 1: **Biochemical characterization and identification of six most promising phosphate solubilizing bacterial isolates based on their 16S rRNA sequencing results and BLAST analysis**

Bacterial isolate	Solubilization index (SI)	Acid phosphatase enzyme specific activity (nmole/ml/mg protein)	Accession number	Bacteria identified on the basis of sequencing results	% Similarity
B1	2.9±0.12	0.833±0.019	KF922482	*Pseudomonas fluorescens*	100%
B2	3.2±0.14	0.984±0.016	KF922483	*Pantoea eucalypti*	99%
B3	3.3±0.1	1.003±0.016	KF922484	*Bacillus thuringiensis*	99%
B4	3.1±0.12	0.842±0.012	KF922485	*Bacillus cereus*	95%
B5	3±0.12	0.769±0.016	KF922486	*Staphylococcus succinus*	100%
B6	3.4±0.13	1.054±0.015	KF922487	*Pseudomonas fragi*	99%

Table 2: **Biochemical characterization and identification of the six most promising phosphate solubilizing fungal isolates based on their sequencing results and BLAST analysis**

Fungal isolate	Solubilization index (SI)	Acid phosphatase enzyme specific activity (nmole/ml/mg protein)	Accession number	Fungi identified on the basis of sequencing results	% Similarity
F1	3.7±0.12	1.057±0.029	KF934405	*Fusarium oxysporum*	96%
F2	3.5±0.129	0.879±0.023	KF934406	*Schizophyllum commune*	99%
F3	3.3±0.1	0.845±0.034	KF934407	*Aspergillus flavus*	99%
F4	3.4±0.12	0.873±0.035	KF934408	*Bipolaris tetramera*	95%
F5	3.2±0.1	0.787±0.015	KF934410	*Alternaria sp. WF166*	97%
F6	2.8±0.12	0.693±0.015	KF934409	*Alternaria brassicae*	100%

Table 3: **Effect of two bacterial isolates exhibiting the best phosphorus release ability on the growth of rice (*Oryza sativa*) plant**

Rice plant characteristics	Uninoculated control plant	Rice plant inoculated with *Pantoe eucalypti*	Rice plant inoculated with *Staphylococcus succinus*	Mixed inoculants (*Pantoe eucalypti* + *Staphylococcus succinus*)	% Increase in plant growth
Height of plant (cm)	51 ± 0.36^{c}	54 ± 0.42^{ab}	55 ± 0.5^{b}	59 ± 0.33^{a}	78.4
Shoot length (cm)	42 ± 0.34^{b}	44 ± 0.53^{b}	45 ± 0.32^{ab}	48 ± 0.28^{a}	70
No. of leaves per branch	9 ± 0.27^{c}	11 ± 0.41^{b}	11.9 ± 0.39^{bc}	15 ± 0.31^{a}	56.2
Root length (cm)	6.0 ± 0.52^{a}	6.3 ± 0.36^{b}	6.6 ± 0.41^{ab}	6.9 ± 0.34^{a}	56.5
Dry weight (g)	2.5 ± 0.41^{c}	$2.6 \pm 0.22b^{c}$	3.1 ± 0.41^{b}	3.5 ± 0.22^{a}	46.2

The chemical fertilizers, if used continuously, may destroy these PGPMs and affect plant health. To complement and eventually substitute mineral fertilizers with biological use of these PGPMs would represent an economically beneficial and ecologically sound alternative.

In moderately fertile soil, phosphate solubilization effect seems to be the most important mechanism involved in plant growth promotion [24]. This is done either by solubilization of inorganic residues which can be done by ion exchange, chelation mechanism, ligand-exchange reactions or by mineralization of organic phosphate using phosphatase enzyme. Thus, PSMs possess one of the most important bacterial physiological traits for plant growth promotion [13, 14]. The plant growth promoting ability of bacterial isolates on rice plant under pot experiment was also investigated at our labs. Two isolates with the best phosphorus release ability, namely, *Pantoe eucalypti* and *Staphylococcus succinus*, were evaluated for their growth promotion ability for rice crop under axenic conditions. Both these isolates were demonstrated to be the two best plant growth promoting rhizobacteria (PGPRs) and their mixed inoculum led to enhanced uptake of soil phosphorus by inoculated rice plants consequently leading to increased plant height, shoot length, number of leaves, root length and plant biomass as compared to uninoculated rice plants (control). The results are

presented in Table 3. It is proposed that these potential inoculants can be mixed in appropriate combinations with chemical fertilizers for sustainable aerobic rice growth promotion strategies.

Phosphate solubilizing fungal nanoparticles and their potential as biofertilizers

Nanotechnology has left no area untouched by its ground breaking scientific innovations. This includes agricultural industry as well. So far, the use of nanotechnology in agriculture has been mostly theoretical, but it has begun and will continue to have a significant effect in the main areas of agricultural industry. Microbial nanotechnology is an emerging field, where microbes can be harnessed for the synthesis of nanomaterials or nanostructures with desirable shapes and sizes. Chemical and physical methods used for the synthesis of nanoparticles over the decades are expensive and various chemicals used for their synthesis are toxic, and hence biological synthesis using microbes (bacteria and fungi) is considered to be more preferred option. The biological synthesis of nanoparticles is associated with ease of availability, non toxicity, quicker synthesis, availability of a variety of potential microbes and cost-effectiveness. Potential applications of microbial nanotechnology provide exciting waves of transformation in agriculture for crop improvement either directly or indirectly. Many evidences for the production of nanoparticles have been reported for various PGPMs such as *Bacillus cereus* [25], *Escherichia coli* [26], *Lactobacillus strains* [27], *Corynebacterium* sp. [28], *Klebsiella pneumoniae* [29], *Fusarium oxysporium* [30] and *Aspergillus fumigatus* [31]. Numerous PGPMs are now commercially available and can act as biofertilizing and / or biocontrol agents. This projected us to use *Bipolaris tetramera* (the most promising phosphate solubilizing fungal isolate in our study) for the extracellular production of silver and gold nanoparticles that have the potential to be conjugated with chemical fertilizers to increase the agricultural productivity. Silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) were analyzed by dynamic light scattering and transmission electron microscopy. AgNPs were irregular shaped with a size ranging between 54.78 to 73.49 nm. AuNPs were spherical or hexagonal, with a size of 58.4 and 261.73 nm, respectively. Some PGPMs serve as key sources of antimicrobial agents in the form of nanoparticles and produce a wide variety of other medicinal compounds such as enzymes, enzyme inhibitors, antitumor agents, insecticide, immune-suppressants and immune-modulators. The prepared nanoparticles were thus analyzed for their antibacterial and antifungal activities. At higher concentration, AgNPs showed effective antimicrobial activity against Bacillus cereus, Staphylococcus aureus, Enterobacter aeroginosa and Trichoderma sp. It is suggested that these myconanomaterials may lead to enhancement of agricultural productivity by allowing slow release of encapsulated acid phosphatase enzyme into soil, which may facilitate the release of phosphorus from soil and chemical fertilizers that can be effectively taken up by the plants.

Conclusion

Phosphorus is an important element in crop nutrition. Adverse environmental effects of chemical based phosphate fertilizers, depleting resources of high-grade phosphatic rocks and their increase in prices have compelled us to find an alternative approach for efficient phosphorus availability for improving crop production to meet the ever-increasing global demand of food. In this direction, the use of efficient PSMs opens up new prospects for healthier crop productivity besides sustaining soil health. Therefore, there is a need for consistent and extensive research efforts to identify and characterize more PSMs with greater efficiency for their ultimate applications under field conditions. This will be achieved through better management of soil microbial communities, by development of more effective microbial inoculants, through the genetic manipulation of specific organisms, or with a combination of these approaches. Further, the myconanoparticles also have the potential to either complement or substitute chemical fertilizers in near future.

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