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Phosphorus is an essential mineral fertilizer for plant growth and development. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems. Plants utilize only a small fraction of the phosphate fertilizers applied to the soil and the rest is rapidly converted into insoluble complexes in the soil. The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. Several studies investigating whole form P budgets have found nutrient P deficits in many organic forms and illustrate the need for nutrient additions. This leads to the need of frequent application of phosphate fertilizers. These fertilizers are expensive, and have some harmful impacts on the soil structure, composition, microflora and other specifications of soil. Chemical phosphate fertilizers use on a regular basis has become a costly affair and also environmentally undesirable. Increasingly high cost of chemical fertilizers has been the major stimulus to search for an alternative, naturally-occurring, dependable, biodegradable, phosphate source. Natural rock phosphates have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of rock phosphate deposits and this material should provide a cheap source of phosphate fertilizer for crop production. Unfortunately, rock phosphate is not readily available to the plants in soils with a pH > 5.5-6.0. Because of this, extension services are reluctant to be recommended and farmers are hesitant to utilize RP directly. One approach for solubilization of rock phosphate in field conditions is the application of phosphate-solubilizing microorganisms (PSMs). PSMs solubilize insoluble form of phosphates by acidification, chelation and exchange reactions and also by production of organic acids. This process not only compensates for higher cost of manufacturing fertilizers in industry; it also mobilizes the fertilizers applied to the soil. In addition to P-solubilization, phosphate-solubilizing microorganisms (PSMs) may also

improve the plant productivity by producing other secondary metabolites. There are several evidences related to plant growth promotion by PSMs through the production of indole acetic acid (IAA) and siderophores. Organic farming avoids the inputs of synthetic chemicals and their consequences. The build-up of a large and active soil microbial biomass is, therefore, critically important for sustaining the productivity of soils in organic farming systems. The use of PSMs with the aim of improving P nutrient availability for plants is an important practice and necessary for agriculture. However, the potential benefits of these PSMs are not fully realized because of the limitations like inconsistent performance at different sites. There is no doubt that bacterial inocula can increase the yield of various crops significantly, but the performance has generally been inconsistent. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. Potential of bacterial inoculums may be determined in a single experiment, but the consistent performance can only be determined in multiple trails. The PSMs occur in soil, usually their number are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase in plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than the normally found in soil is necessary to take advantage of their phosphate solubilization for enhancement of crop yield and soil fertility. Before recommending an organism as bio-inoculants to crop production, its shelf life in different carrier materials needs to be addressed. Aim of present study was to isolate the phosphate-solubilizing microorganisms (bacteria and fungi) from organic farm and evaluate their effect on maize and wheat crop, as bio-inoculants along with RP fertilization in organic farming and at multilocational sites comes under different agroclimatic regions, and compare bio-inoculation and RP fertilization treatment effects with DAP fertilization for the enhancement

of yield and soil fertility. Inoculum formulations were developed with suitable carriers to sustain the shelf life of these inoculants for long period at different temperatures.

Phosphate-solubilizing microorganisms (bacteria and fungi)

To isolate the phosphate-solubilizing microorganisms, soil samples were collected from different agricultural niches. These different niches have three types of fields, one was conventional agriculture field in which all the chemical fertilizers are used by farmers as per agronomic practices, these conventional agriculture fields were bamboo plants (*Bambusa vulgaris*) (multipurpose agroforestry crop) experimental field of CORE, Thapar University, Patiala (30.30° N and 76.38° E); potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; mustard (*Brassica campestris*) crop field (oil-seed crop), mature maize (*Zea mays*) crop field (cereal crop), small maize plants crop field, Berseem (*Trifolium alexandrinum*) crop field (fodder crop); sunflower (*Helianthus annuus*) crop (oil-seed crop) grown in agriculture field of Balachaur (31.07° N and 76.32° E), Punjab. Second type of crop field was organic farm of *Stevia rebaudiana* (medicinal plant) at Pojewal (31.65° N and 76.26° E), Punjab. Organic field used in this study is a field where no chemical fertilizers are used since last ten years. Mainly animal manure, vermi-compost and green manure are used to maintain the soil fertility. And third one was the surface soil of CORE, Thapar University, Patiala that is open land area, without any vegetation.

Phosphate-solubilizing bacteria and fungi were isolated by serial dilution method on Pikovskaya's (PKV) agar plates (Pikovskaya's 1948). Quantitative screening of selected bacterial and fungal isolates was done by the method described by Jackson (1973) in tri-calcium phosphate (TCP) and RP supplemented (equivalent to 100 mg P_2O_5 100 ml^{-1}) PKV broth. Most efficient phosphate-solubilizing bacterial and fungal isolates were come from rhizospheric soil of organic farming. Six bacterial isolates designated as PSB-3, PSB-5, PSB-

6, PSB-7, PSB-12 and PSB-13, isolated from organic farming soil, showed maximum P solubilization up to 429 µg ml^{-1} respectively in TCP amended PKV broth. The main organic acids produced by these selected isolates in TCP solubilization were oxalic acid, citric acid, acetic acid and gluconic acid. These selected bacterial isolates were further screened for solubilization of rock phosphate. Maximum P solubilization of rock phosphate was observed on day five of incubation up to 271 µg ml^{-1} with reduction in pH 3.7 of the medium from its initial value 7.2. The main organic acids produced by these isolates in RP amended PKV broth were oxalic acid and gluconic acid. Production of gluconic acid was higher than that of other organic acids. All the isolates were able to produce significant amounts of acid phosphatase, alkaline phosphatase and phytase enzyme in the TCP and RP amended PKV broth.

Four fungal isolates designated as PSF-4, PSF-5, PSF-6 and PSF-7 showed maximum P solubilization in TCP and RP amended PKV broth were selected for further study. These four fungal isolates produced significant amount of acid phosphatase, alkaline phosphates, phytase enzymes and organic acid in growth medium supplemented with TCP and RP.

Identification of phosphate-solubilizing bacteria and fungi

For the identification of bacterial isolates, 16S rRNA genes were amplified by 16S rRNA primers. The amplicons were purified with QIA gel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per the manufacturer's instructions (Promega Inc., USA). Ligated plasmids were transformed into *Escherichia coli* DH5α cells and recombinant clones with inserts were sequenced. Nucleotide sequences comparison of bacterial isolates was performed using Ez Txzon-e database. Sequence data from Ez Txzon-e database showed that two isolates PSB-3 and PSB-6 showed maximum similarity (98.45 %) with *Pantoea cypripedii* and four isolates PSB-5, PSB-7, PSB-12 and PSB-13 showed similarity (99.85 %)

with *Pseudomonas plecoglossicida*. Out of these six isolates, two isolates PSB-3 and PSB-5 were selected for 16S rRNA full length sequence analysis. 16S rRNA full length sequence analysis using the Ez Txzon-e database revealed that the most closely related type strain for PSB-3 is *Pantoea cypripedii,* which showed 98.67% similarity. For PSB-5, the most closely related sequence is *Pseudomonas plecoglossicida*, with 99.78% similarity. Phylogenetic analysis (Neighbor-Joining Tree) also grouped PSB-3 with *P. cypripedii* and PSB-5 with *P. plecoglossicida*.

The morphological and microscopic identification of fungal isolates reveled that all the nine isolates were belong to genera *Aspergillus*. For molecular identification of fungal isolates ITS region of the genomic DNA of fungal isolates was amplified by using ITS 1 and ITS 4 primers. Results of RFLP analysis of ITS amplified products with restriction enzyme *Rsa1* showed that one isolate PSF-4 does not show any restriction digestion with *Rsa1* and other eight isolates were showed restriction digestion with *Rsa 1*. So on the basis of RFLP analysis PSF-4 was grouped under *Aspergillus tubingensis* and other isolates were grouped under *Aspergillus niger*. Further PSF-4, PSF-5, PSF-6 and PSF-7, which showed maximum P-solubilization, were selected for nucleotide sequencing of ITS region. Nucleotide sequences comparison of fungal isolates was performed using the BLAST database. Sequence data from BLAST results showed that out of four, three fungal isolates (PSF-5 (99 %), PSF-6 (100 %) and PSF-7 (99%)) showed maximum similarity with *Aspergillus niger* and one isolate PSF-4 showed maximum similarity (99 %) with *Aspergillus tubingensis*.

Effect of different carbon and nitrogen sources on P-solubilization of selected bacterial and fungal isolates

To test the effect of different carbon and nitrogen sources on P-solubilization by bacterial (*Pantoea cypripedii* and *Pseudomonas plecoglossicida*) and fungal isolates (*Aspergillus*

tubingensis and *Aspergillus niger*), PKV broth containing different carbon (fructose, arabinose, galactose, mannitol, maltose, lactose, sucrose, xylose and glucose) and nitrogen sources ($NaNO_3$, NH_4NO_3, $NaNO_2$, KNO_3, tryptophan, NH_4Cl, urea and $(NH_4)_2SO_4$) with tri-calcium phosphate (100 mg P_2O_5 100 ml^{-1}) were prepared and inoculated with these isolates. The pattern of P solubilization by *P. cypripedii* in different carbon sources was glucose > xylose > galactose > fructose > arabinose > lactose = sucrose > mannitol > maltose while for *P. plecoglossicida* glucose > xylose > galactose > sucrose > maltose > lactose > fructose > mannitol > arabinose. Main organic acids, produced by *P. cypripedii*, in different carbon sources were oxalic acid, acetic acid and gluconic acid, where as *P. plecoglossicida* produced oxalic acid, citric acid and gluconic acid. *Aspergillus tubingensis*, in presence of different carbon sources, showed pattern of P solubilization as glucose > mannitol > sucrose > xylose > maltose > fructose > galactose > arabinose > lactose. Organic acids produced by *A. tubingensis* in presence of different carbon sources were oxalic acid, succinic acid and gluconic acid. In case of *A. niger* pattern of P solubilization in presence of different carbon sources was glucose > mannitol > maltose > xylose > sucrose > fructose > galactose > arabinose > lactose. Organic acids produced by *A. niger* were oxalic acid, citric acid, malic acid and gluconic acid and maximum acid production was observed in presence of glucose compared to other carbon sources. In both bacterial and fungal isolates, it was observed that acid phosphatase, alkaline phosphatase and phytase enzymes production was higher in presence of glucose compared to other carbon sources in growth medium.

Among the nitrogen sources, ammonium sulphate showed maximum P-solubilization in all the selected bacterial and fungal isolates. In case of *P. cypripedii* pattern of P solubilization was $(NH_4)_2SO_4$ > KNO_3 > NH_4NO_3 > $NaNO_2$ > tryptophan > $NaNO_3$ > NH_4Cl > urea, while in *P. plecoglossicida* the pattern was $(NH_4)_2SO_4$ > urea > tryptophan > KNO_3 > NH_4Cl > $NaNO_2$ > $NaNO_3$ > NH_4NO_3. In *A. tubingensis* P-solubilization pattern was $(NH_4)_2SO_4$ >

KNO_3 > NH_4NO_3 > NH_4Cl > urea > tryptophan > $NaNO_3$ > $NaNO_2$. While in *A. niger* it was $(NH_4)_2SO_4$ > KNO_3 > $NaNO_2$ > $NaNO_3$ > tryptophan > NH_4Cl > urea > NH_4NO_3. *Pantoea cypripedii* produce oxalic acid and gluconic acid as main organic acids in all the nitrogen sources. Organic acids produced by *P. plecoglossicida* in different nitrogen sources were oxalic acid, citric acid, malic acid and gluconic acid. *Aspergillus tubingensis* produced oxalic acid, citric acid, malic acid, succinic acid and gluconic acid, while *A. niger* produced oxalic acid, malic acid and gluconic acid. Acid phosphatase, alkaline phosphatase and phytase enzymes production was higher in presence of ammonium sulphate compared to other nitrogen sources both in bacterial and fungal isolates. It was observed that in both bacterial and fungal isolates, nature of carbon and nitrogen sources affect the P-solubilization. The mechanism of P-solubilization might be due to pH reduction, organic acid production, acid phosphatase, alkaline phosphatase and by phytase enzymes production in both bacterial and fungal isolates.

Plant growth promotion activities of bacterial and fungal isolates

Indole acetic acid (IAA) production was detected in bacterial and fungal isolates with (0.1% L-tryptophan in growth medium) and without addition of L-tryptophan, but the rate of IAA production was higher in presence of L- tryptophan. IAA production in presence of L-tryptophan was found to be higher in case of *P. cypripedii* (92.9 µg ml^{-1}) compared to *P. plecoglossicida* (26.9 µg ml^{-1}). IAA production in presence of L-tryptophan in case of *A. tubingensis* was 50 µg ml^{-1} and in case of *A. niger*, it was 57 µg ml^{-1}. *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were observed to be positive for siderophore production and were negative for hydrogen cyanide production. Both the bacterial and fungal isolates produced hydroxamate type of siderophore.

Effect of buffering on P-solubilization of selected bacterial and fungal isolates

Phosphate solubilization efficiency of selected bacterial and fungal isolates was tested in buffered medium. For this selected isolates were inoculated into Pikovskaya's broth having TCP (equivalent to 100 mg P_2O_5 100 ml^{-1}) and the pH of the growth medium was maintained 7.0, 8.0 and 9.0 with 0.1 M Tris-HCl buffer. P solubilization by selected isolates was compared with un-buffered medium of pH 7.0, 8.0 and 9.0 adjusted with 0.1 M NaOH. Normal PKV broth having TCP (equivalent to 100 mg P_2O_5 100 ml^{-1}) inoculated with *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were kept as control. It was observed that in case of all the isolates, there were no negative effect of buffering of media on P solubilization when compared with control medium and un-buffered medium of different pH ranges. Reduction in pH was observed with increase in soluble P concentration in buffered and un-buffered PKV broth. All the bacterial and fungal isolates were able to produce acid phosphatase, alkaline phosphatase and phytase enzymes in buffered and un-buffered conditions and these enzymes production were comparable with normal broth (control).

Role of phosphate-solubilizing bacteria and fungi in organic farming

A two year field experiment of wheat and maize crop was conducted in organic farming to test the effect of selected bacterial and fungal isolates as bio-inoculants in organic farming alone or along with RP fertilization. All the bacterial and fungal bio-inoculation and RP fertilization treatments were compared with chemical phosphate fertilizer (DAP) treatments and with control treatments. It was observed that there was a significant improvement in plant growth parameters (shoot height, shoot and root dry weight), yield and in total P uptake in both the maize and wheat crop field experiments in all the treatments compared to control soil. Results were more significant and pronounced in inoculation along with RP fertilization

treatments compared to bio-inoculation and DAP treatments. Similarly during the two year of field study, soil fertility was significantly improved in context to organic carbon, total P, available P, soil enzyme activities (acid phosphatase, alkaline phosphatase, phytase enzymes and dehydrogenase enzymes) and phosphate-solubilizing bacterial and fungal population in respective plots compared to control. It was observed that soil total P level was significantly increased with DAP and RP fertilization but available P level was significantly increased only in inoculation treatments along with RP fertilization treatments. After harvesting of maize and wheat crop, reduction in soil pH was observed in bio-inoculation treatments alone or along with RP fertilization compared to control. This may be attributed to ability of phosphate-solubilizing microorganisms to produce organic acids, thereby decreasing the pH and increasing the concentration of phosphorus in soil by mechanism involving chelation and exchange reactions. Results showed that inoculation treatments along with RP fertilization significantly improve the yield and soil fertility in organic farming compared to inoculation alone, RP fertilization alone and DAP treatments. Present study suggested that these phosphate-solubilizing bacterial and fungal strains along with RP fertilization can substitute the chemical fertilizers, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the phosphate solubilizing potential for long time.

P-solubilizing bacteria and fungi at multilocational sites

A two year field experiment was conducted at multilocational sites comes under different agroclimatic regions, to test the consistency in performance of selected phosphate-solubilizing microorganisms (bacteria and fungi), with and without rock phosphate fertilization. Maize and wheat were selected as test crops. Application of both bacterial and fungal isolates showed significant effect on maize and wheat grain yield and on soil fertility at all different agro-climatic regions. Grain yield, P uptake, soil organic carbon, available P

and enzyme activities in all the experimental crop fields at different sites, significantly increased due to inoculation. Improvement in crop yield and soil fertility at three different sites was more pronounced and significantly higher when RP was supplemented along with bio-inoculation compared to inoculation, RP fertilization, DAP treatments. Results of the present study suggest that *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* along with RP fertilization play an important role in plant growth promotion and facilitates improvement in soil fertility in different agro-climatic conditions.

Development of inoculum formulations

Inoculum formulations of *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were developed, where in, rock phosphate, fly ash, charcoal and vermiculite were used as a carrier materials. The final moisture content of the carrier materials were adjusted to 30 % before packing separate bags. Bacterial and fungal population in all carrier materials at 37 °C showed an upward trend up to 15 days of incubation after that it was started decreased in all the carrier materials, while there was no increase in population at 4 °C. Results showed that viability of bacterial and fungal isolates was higher at 4 °C compared to 37 °C. Among the different carriers tested, rock phosphate formulations supported the maximum viability of fugal and bacterial inoculum up to 270 days of storage period at 4 °C and 37 °C compared to other carriers tested. Both bacterial and fungal P-solubilization, enzyme activities and plant growth promotion activities were not affected even after a long storage period of 270 days at different temperatures and were comparable to fresh culture activities. Results showed that RP can be used as a carrier material for the development of inoculum formulations of selected bacterial and fungal isolates that maintain the viability and P solubilization efficiency of selected isolates up to 270 days of storage at 4 ºC and 37 ºC. Rock phosphate may be the best carrier material for PSMs due to its low cost, positive effects on crop yield and on improvement of soil fertility on its amendments in soil.