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Isolation of phosphate solubilizing microorganisms and their effect on seed germination and growth of *Pisum sativum*

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Abstract
The aim of this study was to isolate phosphate solubilizing microorganisms from soil samples in and around MES College, Marampally and evaluate their effect on seed germination and growth of *Pisum sativum* under constant observation. A total of 23 phosphate solubilizing microorganisms were isolated from 50 samples collected randomly from rhizosphere soil and root nodules. The phosphate solubilizing microorganisms were selected based on their solubilisation index on Pikovskaya's medium (PVK) and phosphatase assay. Two isolates named as PSM01 and PSM21 showed highest amount of phosphate solubilisation and were selected for further studies. PSM01 and PSM21 were identified as *Aspergillus niger* and *Aspergillus fumigatus* respectively. Their inoculation onto *Pisum sativum* increased the percentage of seed germination (75%), amplified radicle and plumule lengths of germinated seeds, increased mean shoot lengths and root lengths and vigour index (513.75) when compared to non-inoculated ones. On seventh day, plumule and radicle length of *A. niger* and *A. fumigatus* treated *P. sativum* were 3.8 and 4.8 cm respectively. On statistical analysis P value in case of *A. fumigatus* and *A. niger* is found to be < 0.05, indicating the effect is extremely significant. These two *Aspergillus* sp. act as efficient phosphate solubilizer and can be considered as a substitute for chemical fertilizers in agricultural systems, after evaluation of its performance and environmental impact by proper field trial, thereby functioning as an ideal bio fertilizer.

Keywords: *Aspergillus niger*, *Aspergillus fumigatus*, *Pisum sativum*, phosphate solubilizing microorganisms, seed germination

Introduction
Phosphorous (P), the second most significant macronutrient for plant growth, is involved in a variety of physiological processes such as respiration and photosynthesis, energy transfer mechanisms, cell proliferation, seed generation, and root system development. Nucleic acids, phytin, and phospholipids all contain it as a structural component. It also enhances stalk and stem strength, bloom maturity, and higher-quality crop production. Phosphorous is also reported to protect plants against a variety of illnesses. However, the majority of phosphorus in soil (up to 95-99 percent) is contained as insoluble compounds, making it unavailable to plants. Phosphorus deficiency causes the leaves to turn brown, resulting in the generation of tiny leaves, a weak stem, and poor growth.

Researchers have recognised the ability of soil microbes to solubilize solid phosphate compounds to accessible orthophosphate more than a century later (Pi). Plants and microorganisms use an enzyme called "phosphatase" to assimilate phosphate from organic substances. Phosphatase is found in a wide spectrum of microbial species. As an outcome, these microbes are responsible for a large portion of the overall recycling of insoluble molecules. The mechanism for phosphate biosolubilization present in a lot of soil fungus and bacteria [1-5]. Phosphate Solubilizing Microorganisms is the way of referring to them all (PSMs). Among the bacterial genera with similar qualities are *Pseudomonas*, *Azospirillum*, *Bacilus*, *Rhizobium*, *Arthrobacter*, *Serratia*, *Flavobacterium*, and *Erwinia* [6]. Bacteria are much more successful than fungi in phosphorus solubilization [7] and are localised in the rhizosphere. Phosphate is solubilized by a number of microbiological activities, including the production of organic acids.

PSM (bio fertilisers) are among the most effective plant aids for supplying phosphorous at a suitable level. The introduction of these microbes into the root activity zone aids in the plant's ability to absorb more nutrients. Crop yields can be increased by up to 70% when PSMS is used. PSM improve plant growth and seedling length by increasing the availability of soluble phosphate. Despite the fact that PSMs are abundant in many soils, isolation, identification, and selection of PSMs have yet to be marketed. As a consequence, the application is still considered limited. Hence the goal of this research is to isolate and identify phosphate-solubilizing microorganisms in soil samples. The next step is to investigate its impact on *Pisum sativum* seed germination and seedling growth, as well as the growth of these plants in pots inoculated with these isolated PSMs for two months.

Materials and Method

Isolation of phosphate solubilising microorganism

The soil samples were taken in and around MES College, Marampally, from various rhizosphere soils. The samples were collected in aseptic polythene bags and kept in the laboratory for further analysis. The relevant bacteria were isolated using the traditional serial dilution approach. To prepare an initial dilution, one grams of rhizosphere soil was suspended in 9 ml of distilled water from each sample obtained. To acquire an adequate dilution factor, a ten-fold serial dilution was performed. 0.1ml of aliquots from the appropriate serial dilution were dispersed on petriplates containing Pikovskaya's Agar Medium (PVK Medium). For two to seven days, the plates were incubated at 32°C. By spot inoculating near the centre of another Pikovskaya's plate and incubating at 37°C, all colonies with halozones were screened for phosphate solubilisation on Pikovskaya's medium. The clearing zone's diameter was measured every 24 hours for up to seven days. All observations were made three times. The PSI (Phosphate Solubilization Index) is the ratio of the colony diameter to the total diameter (clearing zone plus fungal growth).

$$\text{PSI}= \frac{\text{COLONY DIAMETER +HALOZONE DIAMETER}}{\text{COLONY DIAMETER}}$$

Analysis of phosphate solubilising activity

From the isolates, larger halo zone producing strains were selected and. The quantitative estimation of phosphatase activity of microbial isolates was done by the vanadomolybdophosphoric yellow colour method [8].

Identification of isolated organisms

Lacto phenol cotton blue staining technique was used for identification of isolated fungal cultures.

Application studies of isolated PSM

Preparation of conidial suspension of fungal culture

The phosphate solubilizing fungus, *Aspergillus* sp. was mass multiplied by inoculating into Sabroaud dextrose agar medium (SDA) and incubating at 25^0C for seven days. After incubation, an aliquote of 10ml of sterile distilled water was added to each of the culture plates and gently shaken to dislodge conidia from the culture surface and collected in 250 ml conical flask and further centrifuged. The resulting pellets were re-suspended in sterile distilled water and the concentration of conidia was adjusted to 1×10^8 CFU/mL using haemocytometer.

Assay of seed germination and seedling growth

Seed germination bioassay is usually done by calculating the vigour index of germinating seeds. This method examines whether inducer treatment is successful in increasing seed vigour and rate of germination. Here, we compared the two inducer treated (*Aspergillus niger* and *Aspergillus fumigatus*) to that of the untreated control seeds. The seeds of *Pisum sativum* were surface sterilized with 0.25% sodium hypochlorite for about 2minutes and rinsed thoroughly in sterile distilled water for 2-3 times. The sterilized seeds were treated with conidial suspension and culture filtrate of phosphate solubilizing fungi at 1×10^8 CFU/mL and kept overnight in the laminar flow hood for drying. A control set was kept by using seeds treated with sterile distilled water (control). The inducer treated and control seeds (four replicates of four seeds each) were placed equidistantly on a layer of moistened Whatman no. 1 filter paper disc in petridishes to evaluate percentage germination. Subsequently, the percentage of rate of seed germination was noted for 1-3 days, radicle and plumule length of germinated seeds were measured up to 7 days. The vigour index of seeds was determined using the following formula,

$$\text{Germination Percentage} = \frac{\text{No: of Seeds Germinated } \times 100}{\text{No: of Seeds Sown}}$$

$$\text{Vigour Index} = \text{Seed Germination}(\%) \times [\text{Mean Radicle Length} + \text{Mean Plumule Length }]$$

Assay of plant growth upto two months in pots

Pot experiment was carried out to investigate the effects of phosphate solubilizing fungus with single inoculation on *Pisum sativum* growth [9]. Four seeds were sown into each pot after 7 days of emergence and maintained for two months. The plants were watered at regular intervals and ensured enough sunlight and presence of other nutrients.

Results

Isolation of microorganisms

Out of the 50 samples, 23 samples showed colony formation and exhibited halozone on PVK. Phosphate solubilising index (PSI) of ten strains was shown in table 1. PSM01 and PSM21 showed highest PSI, 1.66 and 1.89 respectively.

Table 1: Phosphate Solubilising Index (PSI) of top strains

Fungal Strains Showing Halozone	Phosphate Solubilising Index (PSI)
PSM01	1.66±0.12
PSM02	1.21±0.02
PSM03	1.06±0.22
PSM07	1.45±0.04
PSM08	1.38±0.17
PSM14	1.46±0.18
PSM17	1.21±0.14
PSM18	1.35±0.46
PSM19	1.46±0.42
PSM21	1.89±0.38

Analysis of phosphate solubilising activity

Phosphate solubilising activity of fungal strains was shown in Fig.1. PSM01 and PSM21 showed higher phosphate solubilising activity (PSA) compared to other strains and these were selected for further studies.

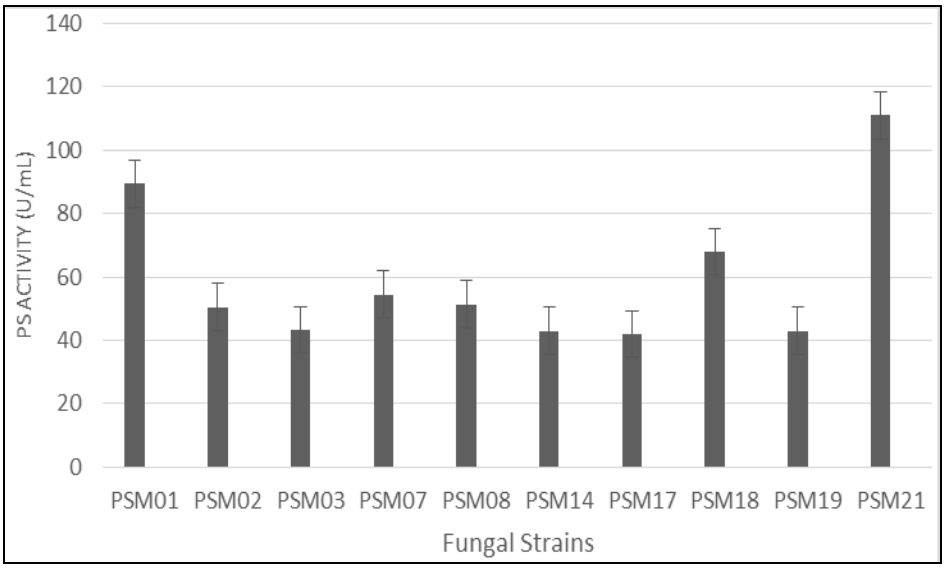

Fig 1: Phosphate solubilising activity of fungal strains

Identification of PSM

Colony characteristics

PSM01 was shown in Fig.2: colonies were velvety or powdery, white at first then fuming to smoky green, greyish green. Reverse side is white to tan.

PSM21 was shown in Fig.3: colonies were white to pale yellow in colour, wooly, turning dark brown to black due to profuse conidial formation. Reverse side is white to yellow.

Fig 2: Clear zone indicating PSI of PSM 01

Fig 3: Clear zone indicating PSI of PSM21

Staining technique (LPCB): Careful analysis of hyphae morphology and conidial arrangement was done. Septate hyphae with conidiophores arising from fungal cells seen. Single celled spores arise at the terminal end of conidiophores. Fig. 5 and 6 showing microscopic view of PSM01 and PSM21 respectively. Based upon the study of colony characteristics and the staining results, PSM01 was identified as *Aspergillus fumigatus* and PSM21 was identified as *Aspergillus niger.*

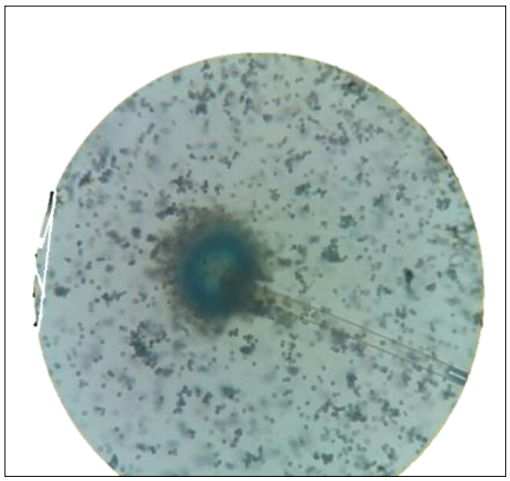

Fig 4: Microscopic view of PSM01

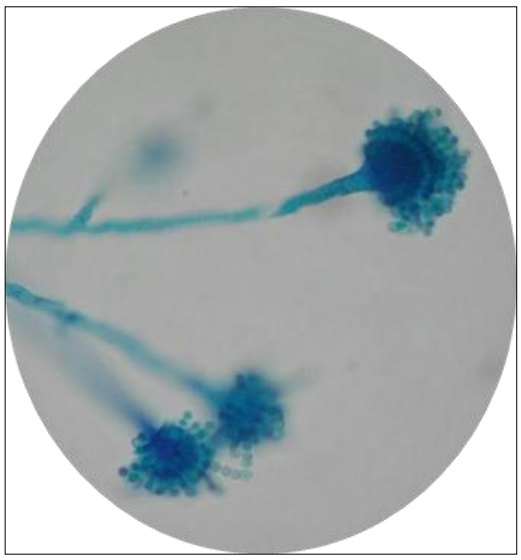

Fig 5: Microscopic view of PS

Application studies of the isolated PSM

Fig. 7, 8 and 9 shows the germination of seeds after 4th day of inducer treatment.

Fig 6: Seeds treated with conidial suspension of A. fumigatus

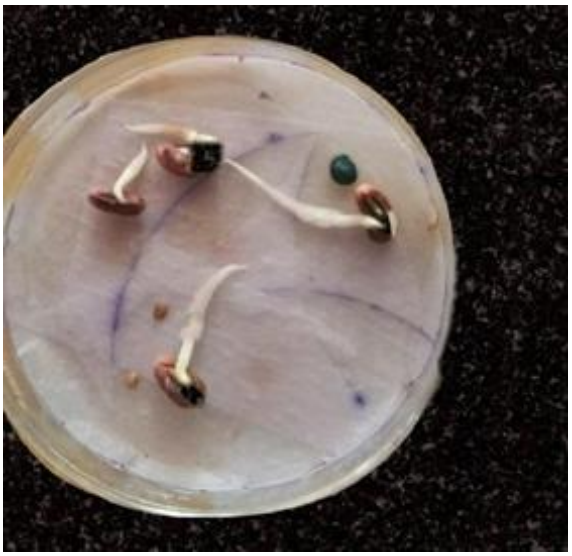

Fig 7: Control set (untreated)

Fig 8: Seeds treated with conidial suspension of A. niger

Seed germination assay

The results of this study was shown in the table 2. Germination percentage, plumule and radicle lengths and vigour index were calculated and noted as follows, conidial suspension and culture filtrate of the phosphate solubilizing fungi *Aspergillus* sp., were used as seed treatment to *Pisum sativum* plants. In comparison with the control, a significant enhancement of seed germination and vigour was noticed in phosphate solubilizing fungi *Aspergillus* sp, with maximum germination of 75% and seedling vigour index score of 513.75. On seventh day, plumule and radicle length of *A.niger* and *A.fumigatus* treated *P.sativum* were 3.8 and 4.8 cm respectively.

On statistical analysis P value in case of *A. fumigatus* and *A. niger* is found to be < 0.05, indicating the effect is extremely significant.

Table 2: Table for seed germination assay

Plate no:	Plumule length		Radicle length		Seed germination (%)	Vigour index
	On fourth day (cm)	On seventh day (cm)	On fourth day (cm)	On seventh day (cm)		
A.niger	2.1±0.42	3.8±0.16	3.0±0.47	4.8±0.18	75	513.75
A.fumigatus	1.8±0.36	2.6±0.11	2.4±0.56	3.4±0.16	75	392.5
Control	0.9±0.12	1.4±0.32	1.6±0.43	2.1±0.26	50	157.5

Assay of plant growth upto two months in pots

The results of pot experiment and growth of *P. sativum* inoculated with *A. niger* and *A. fumigatus* isolates were presented in the table 3. All of the inoculated treatments showed increased plant height when compared to the control set. Among the treated sets, *A. niger* gave maximum plant growth followed by *A. fumigatus*. Mean root and shoot length of *A. niger* treated plants increased to 28 cm and 9 cm respectively. Most of the plants inoculated with *A. niger* and *A. fumigatus* produced more number of leaves when compared to the non-inoculated control set. On statistical analysis P value in case of *A. fumigatus* and *A. niger* is found to be < 0.05, indicating the effect is extremely significant.

Table 3: Table for observation of plant growth (P. sativum) after two months of pot experiment

Pot NO:	No: of Leaves	Mean Root Length (cm)	Mean Shoot Length (cm)
A. niger	52±3	28±1.2	9±2.1
A. fumigatus	30±5	24±0.4	7.2±0.5
Control	18±2	11±0.2	4.3±0.2

Fig 9: Observation of control

Fig 10: Observation of plant treated with A. fumigatuse

Fig 11: Observation of plant treated with A. Niger

Discussion

In terms of numerical plant requirements, phosphorus is the 2nd largest critical element behind nitrogen. Although phosphates are abundant in soils, their availability is limited since they are usually insoluble. P is commonly applied to land as synthetic P fertiliser to meet agricultural nutritional needs. However, chemical P fertiliser synthesis is a high-energy process with long-term environmental consequences in terms of

eutrophication, soil fertility depletion, and carbon footprint. As a result of these environmental problems, researchers are looking for a long-term solution for crop P nutrition. Phosphate-solubilizing microorganisms (PSM) have been found as the most environmentally benign method for crop P feeding.

There are various types of soil microbes which can solubilize this fixed form of P and make it available to plants by improving biological nitrogen fixation [10-12]. Although various fungal strains have been identified as PSMs, their performance in situ is unreliable, necessitating the use of genetically modified strains or co-inoculation strategies to increase their performance.

A total of 23 fungi were isolated from 50 samples of rhizosphere soil and tested for their ability to solubilize phosphates in PVK medium in this study. Only *Aspergillus* sp, PSM01, and PSM21 were reported to be positive for phosphate solubilization among the 23 rhizosphere fungus. Similarly, fungal isolates that display P solubilizing activity by forming a clear halo zone (indication of solubilization) around their colonies have been reported. Mendes *et al*., 2013 discovered that isolates of *Aspergillus niger* FS1, *Penicillium canescens* FS23, and *Eupenicillium ludwigii* were capable of solubilizing all types of P [13]. Phosphate-solubilizing microorganisms that showed better in vitro solubilization (both qualitatively and quantitatively) of insoluble P were chosen for field testing prior to bulk production for final transmission as a bio fertilizer. It's also possible that the fungus's phosphate solubilization, synthesis of IAA, and other similar substances will interact with plants during colonisation, promoting growth, inducing resistance, and modifying basal plant defence mechanisms.

In addition, conidial suspension and culture filtrate of the phosphate solubilizing fungus *Aspergillus* sp. were utilised as seed treatments for *Pisum sativum* plants in this investigation. Seed germination and vigour were significantly improved in phosphate solubilizing fungi *Aspergillus* sp. as compared to the control, with maximum germination of 75% and a seedling vigour index score of 513.75.Tomato seeds treated with phosphate-solubilizing fungi such as *Aspergillus awamori* and *Trichoderma viride*) enhanced seed germination and seedling vigour [14].

In comparison to the control, the co-inoculants enhanced radicle and plumule length. The plumule and radicle lengths of *P.sativum* treated with *A.niger* and *A.fumigatus* were increased by 2 fold when compared to control on the seventh day. This finding is consistent with Sharma *et al*., who found that the PSB co-inoculants (*P. fluorescens* and *B. megaterium*) increased radicle and plumule length [15]. In addition, Balamurugan *et al*., (2010) found that co-inoculation of PSB increased wheat radicle and root length when compared to individuals [16]. This could be due to inoculants releasing a higher amount of growth-promoting chemicals. Both isolated PSMs have the ability to increase seed germination in addition to phosphate solubilizing capabilities.

The selected inoculants considerably increased the growth of *P. sativum* in the pot experiment when compared to the non-inoculated plant (control set). Among the treatments, *A. niger* outperformed the others in terms of increasing shoot height. This could be attributed to increased P-solubilizing and mineralizing abilities from P-sources, as well as the creation of growth-promoting chemicals like IAA. Co-inoculants increased the number of leaves on *P. sativum*.The synergistic action of co-inoculants for the release of growth chemicals as well as mineralization and solubilization of P-sources could explain the rise in leaf number [17].

Conclusion

When compared to the control group, the inoculants (PSM01 and PSM21) dramatically improved seed germination, radicle, and plumule length. Plant biomass, as measured by root length, shoot length, and leaf number, increased significantly. When treated with the conidial suspension of these phosphate solubilizing microorganisms, seed germination and crop plant vigour were improved [17].

References

1. Duponnois R, Kisa M, Plenchette C. Phosphate solubilizing potential of the nematofungus Arthrobotrys oligospora. J. Plant Nutr. Soil Sci,2006:169:280-282.
2. Hassan TU, Bano A. The stimulatory effects of L-tryptophan and plant growth promoting rhizobacteria (PGPR) on soil health and physiology of wheat. J. Soil Sci. Plant Nutr,2015:15:190-201.
3. Rfaki A, Nassiri L, Ibijbijen J. Phosphatesolubilizing bacteria in the rhizosphere of some cultivated legumes from Meknes region, Morocco. Brit. Biotechnol. J,2014:4:946.
4. Son HJ, Park GT, Cha MS, Heo MS. Solubilization of insoluble inorganic phosphates by a novel salt-and pH-tolerant Pantoea agglomerans R-42 isolated from soybean rhizosphere. Bioresource Technol,2006:97:204-210.
5. Whitelaw MA. Growth promotion of plants inoculated with phosphate-solubilizing fungi, in: Sparks, D.L. (Ed.), Advances in Agronomy. Academic Press, Waltham, MA, 1999, 99-151.
6. Rodriguez L, Contreras-Cornejo H, Cortes-Penago, Lopez-Buci, J. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiology,1996:149:1579-1592.
7. Alam S, Khalil S, Ayub N, Rashid M. In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganism (PSM) from maize rhizosphere. Intl. J. Agric. Biol,2002:4:454-458.
8. Subbarao NS. Phosphate solubilizing microorganism. In: Biofertilizer in agriculture and forestry. Regional Biofert. Dev. Centre, Hissar, India, 1988, 133-142.
9. Kucey RMN, Janzen HH, Legget ME. Microbial mediated increases in plant available phosphorus. Adv. Agron,1989:42:199-228.

10. Ponmurugan P, Gopi C. Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. J. Agron,2006:5:600-604.
11. Illmer P, Schinner F. Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol. Biochem,1995:24:389-395.
12. Richardson AE. Prospects for using soil microorganisms to improve the acquisition of phosphorous by plants. Australian Journal of Plant Physiology,2001:28:897-906.
13. Mendes GO, Dias CS, Silva IR, Júnior JI, Pereira OL, Costa MD. Fungal rock phosphate solubilization using sugarcane bagasse. *World journal of microbiology & biotechnology*,2013:29(1):43-50.
14. Sibi G. "Role of phosphate solubilizing fungi during phosphocompost production and their effect on the growth of tomato (Lycopersicon esculentum L) plants." *Journal of Applied and Natural Science*,2011:3(2):287-290.
15. Sharma K, Dak G, Agrawal A, Bhatnagar M, Sharma R. Effect of phosphate solubilizing bacteria on the germination of Cicer arietinum seeds and seedling growth. J. Herb Med. Toxicol,2007:1:61-63.
16. Balamurugan A, Princy T, Pallavi RV, Nepolean Jayanthi PR, Premkumar R. Isolation and characterization of phosphate solubilising bacteria in tea (*Camellia sinensis*) J Bio Sci Res,2010:4(4):285-293.
17. Kumara TA, Shahsavani S, Nezarat S. The effect of Plant Growth Promoting Rhizobacteria (PGPR) on germination, seedling growth and yield of maize. World Acad. Sci. Eng. Technol,2009:49:19-24.