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Isolation of phosphate solubilizing fungi from rhizosphere soil and its effect on seed growth parameters of different crop plants

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ABSTRACT

A total of twenty two rhizospheric fungi were isolated from different rhizosphere soil of healthy crop plants of Mysore, Karnataka, India and tested for their efficacy to solubilize phosphates in PKV medium. Among the isolates, *Penicillium* sp. RFUOM 14 solubilized phosphates by producing clear halo zone around the colony. Further, *Penicillium* sp. RFUOM 14 inducers were treated for different crop plants and observed that the treatments enhanced seed germination and seedling vigor. Among the treatments, conidial suspension treated for 6 h showed maximum of 85.75 %, 80 % and 83 % of seed germination and seedling vigor 985.25, 523 and 673.5 in pearl millet, brinjal and tomato, respectively.

1. INTRODUCTION

Phosphorous (P) is an essential macronutrient for plant growth. It is one of the major limiting factors for crop production on many tropical and sub tropical soils [1]. It plays an important role in energy transfer reactions, respiration and photosynthesis. In plants, phosphorous increases the strength of cereal straw, promotes flower formation and fruit production, stimulates root development and also essential for seed formation [2]. It also plays a role in root development, stalk and stem strength, flower and seed formation, maturity and production, crop quality and resistance to plant diseases. There are evidences of occurrence of rhizospheric phosphorous solubilizing microorganisms which play a key role in soil phosphorous dynamics and subsequent availability of phosphorous to plants [3].

Although large amounts of soluble phosphates are applied to soil as fertilizers, plants are able to use only a small portion of the applied phosphate and the remains are rapidly immobilized and becomes unavailable to plants. Many soil fungi and bacteria are known to solubilize inorganic phosphate and are termed as Phosphate Solubilizing Microorganisms (PSMs). These PSMs play an important role in supplementing phosphorous to the plants, allowing a sustainable use of phosphate fertilizers [4]. Since, plants can absorb phosphate only in soluble form; the transformation of insoluble phosphate into soluble form is carried out only by microbes present in the soil. Several groups of microorganisms including fungi, bacteria and actinomycetes are known as efficient fixed P solubilizers [5].

Fungi are the important components of soil microbes typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. Fungi have been reported to have greater ability to solubilize insoluble phosphate than bacteria [6]. A wide range of soil fungi are reported to solubilize insoluble phosphorous such as *Aspergillus niger* and *Penicillium* sp., which are the most common fungi capable of phosphate solubilization [7]. For a sustainable agricultural system, microbial solubilization of rock phosphate and its use in agriculture is receiving greater attention. Many soil fungi and bacteria can solubilize inorganic phosphate into soluble forms through the process of acidification, chelation, exchange reactions and production of organic acids [8]. Application of PSMs in the field has been reported to increase crop yield. Chemical fertilizers pose health hazard and affect the microbial population in soil by degrading the physical structure of the soil leading to lack of oxygen in the plant root zone besides being quite expensive and making the cost of production high.

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Whereas naturally, the majority of the microorganisms distributed around plant root surface have a role in the decomposition of organic matter and some way suppress deleterious microorganisms distributed around plant root surface and have role on uptake of nutrients and decomposition of organic matter [9]. Hence, the main objective of the present study was to isolate phosphate solubilizing fungi from rhizosphere and to test their ability to increase seed germination and seedling vigor of pearl millet, tomato and brinjal plants.

2. MATERIALS AND METHODS

2.1. Collection of soil sample

The rhizospheric soil samples were collected from different regions of Mysore district of Karnataka from various crop plants. The collected soil samples were brought into laboratory, dried and used for further studies.

2.2. Isolation and identification of rhizosphere fungi

Each of the rhizosphere soil samples were subjected for serial dilution on potato dextrose agar (PDA) medium and incubated for 7 days at 25 °C. After 7 days of incubation, each individual fungal colony was picked from the edge with a sterile fine tipped inoculation needle and transferred on to the PDA medium [10]. The fungi were identified based on the morphological, conidial (fruiting bodies) and culture characters. The classifications of the fungi were carried out following standard procedures. All the isolated fungi were named and maintained in test tubes and Petri plates containing PDA media and used for further studies.

2.3. Screening of phosphate solubilizing fungi

In order to detect phosphate-solubilizing fungi, each of the isolated rhizosphere fungi were inoculated on Pikovskaya's agar (PVK, containing per litre: 0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_2O$ and 15 g agar, pH 7.2). The plates were incubated at 25±2° C for 10 days [11]. Fungi capable of producing a halo/ clear zone due to solubilization were selected as potential phosphate solubilizers and used for further studies.

2.4. Preparation of inducers from *Penicillium* sp. RFUOM 14

2.4.1. Conidial suspension (CS)

Phosphate solubilizing fungus *Penicillium* sp. RFUOM 14 was mass multiplied on PDA medium and incubated at 25±2 °C for 7 days. After incubation, an aliquot of 10 ml of sterile distilled water (SDW) 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. The collected conidial suspension were further passed through a few layers of cheese cloth and centrifuged. The resulting pellate was re-suspended in SDW and the concentration of conidia was adjusted to 1×10^8 cfu/ ml using Haemocytometer [12].

2.4.2. Culture Filtrate (CF)

Mycelial discs (10-15 numbers; 5 mm diameter each) of Phosphate solubilizing fungus *Penicillium* sp. RF UOM 14 grown on PDA medium obtained from the actively growing margins were transferred to 500 ml conical flasks containing 200 ml of potato dextrose broth (PDB) (pH 6.5). The conical flasks inoculated with *Penicillium* sp. RFUOM 14 was incubated at 25±2 °C for 10-12 days. The resulting culture filtrate was filtered through few layers of cheesecloth and the filtrate was centrifuged at 8000 rpm for 15 min. The supernatant was collected and sterilized by filtration through 0.22 µm Millipore membrane. The sterile filtrates were collected and stored at 4 °C [13].

2.5. Inducer treatment

The seeds of pearl millet, brinjal and tomato were surface-sterilized with sodium hypochlorite for 2 min, and rinsed thoroughly in sterile distilled water (SDW) 2-3 times. The sterilized seeds were treated with conidial suspension and culture filtrate of phosphate solubilizing fungi *Penicillium* sp. RF UOM 14 at $1x10^8$ cfu/ ml and culture filtrate by mixing 400 seeds of each of the samples with 50 ml conidial suspension. Treated seeds were kept at 25±2° C in a rotary shaker for 3 h and 6 h separately to facilitate the penetration of the inducer. After incubation, the seeds were air dried aseptically under laboratory conditions (25±2° C) and used for further studies. Seeds treated with SDW served as untreated control.

2.6. Effect of seed treatment with inducers of *Penicillium* sp. RFUOM 14 on seed germination and seedling vigor

The inducer treated and untreated seeds (four replicates of 100 seeds each) were plated equidistantly on three layers of moistened blotting paper discs in Petri dishes to evaluate percentage germination [14] and another set of treated and untreated seeds were subjected to between paper method to record seedling vigor [15]. The experiment consisted of four replications of 100 seeds (50 seeds in eight towels) and was repeated thrice. After 7 days (pearl millet) and 14 days (for brinjal and tomato), percentage germination and vigor were calculated using the formula:

Vigor index = Seed germination (%) × [Mean Root Length + Mean Shoot Length]

2.7. Statistical analysis

Data from four replicates were analyzed for each experiment and subjected to arcsine transformation and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by F values ($P < 0.05$). Treatment means were separated by Tukey's honestly significant differences (HSD) test.

3. RESULTS

3.1. Isolation and identification of rhizosphere fungi

A total of 22 rhizospheric fungi were isolated from the rhizosphere soil of different healthy crop plants of Mysore district of Karnataka, India (Table 1). The isolated fungi belonged to the genera of *Aspergillus*, *Fusarium*, *Nigrospora*, *Penicillium*, *Pestalotiopsis* and *Trichoderma*. All the isolated rhizosphere fungi were serially numbered (RFUOM 01 to RFUOM 22) and maintained on Petri plates and test tubes containing PDA medium.

Table 1: Fungi isolated from the rhizosphere soil of different crop plants

Rhizosphere fungi	Place of collection				No. of isolates
	T. Narsipur	H.D. Kote	K.R. Nagar	Hunsur	
Aspergillus sp.	0	2	1	1	04
Fusarium sp.	1	1	1	2	05
Nigrospora sp.	1	-	2	-	03
Penicillium sp.	2	1	1	1	04
Pestalotiopsis sp.	-	1	-	-	02
Trichoderma sp.	2	1	-	1	04
Total	06	06	05	05	22

Figures inside the column represent the total number of rhizosphere fungal isolates

3.2. Screening of phosphate solubilizing fungi

All the isolated rhizosphere fungi were sub cultured on Pikovskaya's agar medium to test their ability to solubilize the organic phosphate in the medium. Among the tested fungal isolates, *Penicillium* sp. RFUOM 14 was capable of producing a halo/ clear zone due to solubilization of phosphate (Fig. 1), while all the other fungal isolates were unable to produce clear/ halo zone in the PKV medium.

3.3. Effect of seed treatment with inducers of *Penicillium* sp. RFUOM 14 on seed germination and seedling vigor

Seeds treated with inducers of phosphate solubilizing fungus *Penicillium* sp. RFUOM 14 were analyzed for their effect on seed germination and seedling vigor of selected crop plants. The treatment of conidial suspension and culture filtrate of *Penicillium* sp. RFUOM 14 to the seeds of different crop plants enhanced the seed germination and seedling vigor when compared to respective control sets.

Among the different time intervals of treatment, inducers treated for 6 h offered maximum enhancement in seed germination and seedling vigor when compared to 3 h treated seedlings.

A maximum of 86%, 80% and 83% of seed germination and seedling vigor 985.25, 523 and 673.5 was observed in pearl millet, brinjal and tomato seedlings, respectively when treated as conidial suspension for 6 h (Table 2 & 3). The culture filtrate treatment of *Penicillium* sp. RFUOM 14 also enhanced seed germination and seedling vigor but was lower when compared to conidial suspension treatment for 6 h (Fig. 2).

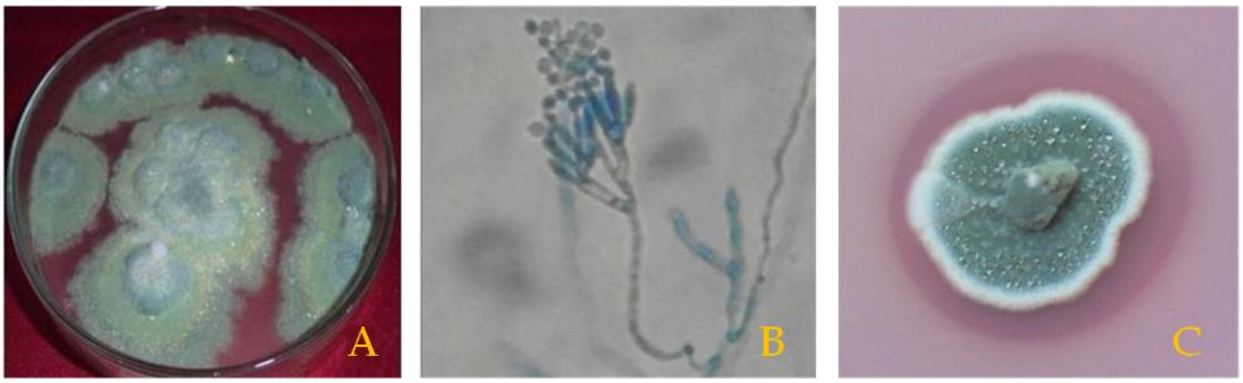

Fig 1: Phosphate solubilizing *Penicillium* sp. RFUOM 14. A- Colony morphology; B- Conidia under Microscope; C- Zone of phosphate solubilization on Pikovskaya plates.

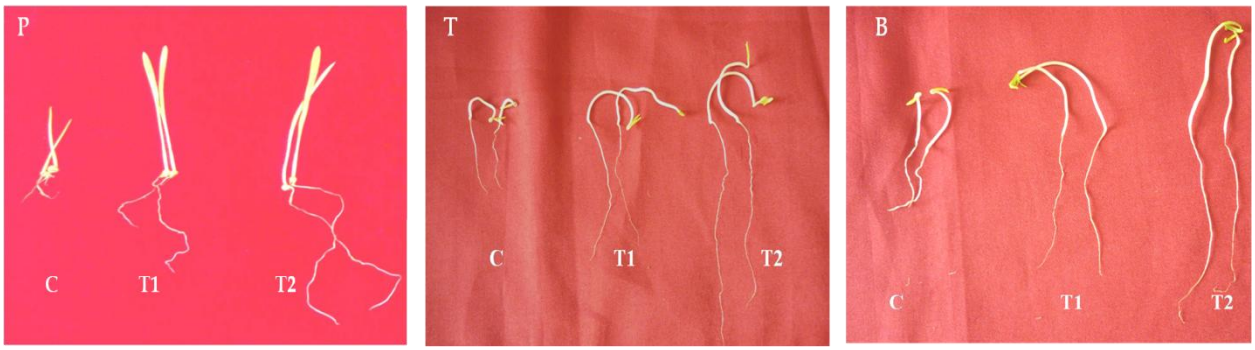

Fig 2: Seed treatment with *Penicillium* sp. RFUOM 14 on seed germination and vigor. P-Pearl millet; T- Tomato; B- Brinjal; C- Control; T1- CF; T2- CS.

Table 2: Effect of seed treatment with inducers *Penicillium* sp. RFUOM 14 on seed germination and vigor after 3 h.

Fungi	Treatment	Pearl millet		Brinjal		Tomato	
		SG	SV	SG	SV	SG	SV
Penicillium sp.	Conidial suspension	81.25 ± 0.47[a]	847.25 ± 5.43[a]	75.00 ± 0.81[a]	488.50 ± 6.02[a]	81.00 ± 0.70[a]	670.00 ± 4.84[a]
RF UOM 14	Culture Filtrate	78.25 ± 0.62[b]	803.50 ± 4.78[b]	72.00 ± 0.81[b]	460.50 ± 4.19[b]	79.50 ± 1.04[b]	641.50 ± 4.19[b]
Control		72.75 ± 0.85[c]	763.25 ± 6.01[c]	70.00 ± 0.40[b]	440.50 ± 3.86[c]	77.00 ± 0.81[c]	620.50 ± 4.64[c]

Values are means of four independent replicates. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD.

Table 3: Effect of seed treatment with inducers of *Penicillium* sp. RFUOM 14 on seed germination and vigor after 6 h.

Fungi	Treatment	Pearl millet		Brinjal		Tomato	
		SG	SV	SG	SV	SG	SV
Penicillium sp.	Conidial suspension	85.75 ± 0.62[a]	985.25 ± 4.49[a]	80.00 ± 0.70[a]	523.00 ± 2.48[a]	83.00 ± 0.81[a]	673.50 ± 3.17[a]
RF UOM 14	Culture Filtrate	80.00 ± 0.81[b]	962.00 ± 4.32[b]	74.00 ± 0.81[b]	492.00 ± 4.32[b]	80.00 ± 0.70[b]	662.00 ± 3.36[a]
Control		72.75 ± 0.85[c]	763.25 ± 6.01[c]	70.00 ± 0.40[c]	440.50 ± 3.86[c]	77.00 ± 0.81[c]	620.50 ± 4.64[b]

Values are means of four independent replicates. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD.

4. DISCUSSION

Phosphorus is the second important key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. Although phosphates are abundant in soils, in both organic and inorganic forms, its availability is restricted as it occurs mostly in insoluble forms. To satisfy crop nutritional requirements, P is usually added to soil as chemical P fertilizer, however synthesis of chemical P fertilizer is highly energy intensive processes and has long term impacts on the environment in terms of eutrophication, soil fertility depletion, carbon footprint. Moreover, plants can use only a small amount of this P since 75–90% of added P is precipitated by metal cation complexes, and rapidly becomes fixed in soils [16]. Such environmental concerns have led to the search for sustainable way of P nutrition of crops. In this regards phosphate-solubilizing microorganisms (PSM) have been seen as best eco-friendly means for P nutrition of crop. Although, several bacterial (*Pseudomonads and Bacilli*) and fungal strains (*Aspergillus* sp. and *Penicillium* sp.) have been identified as PSMs [16, 17] and their performance under *in situ* conditions is not reliable and therefore needs to be improved by using either genetically modified strains or co-inoculation techniques.

In the present investigation, fungi were isolated from rhizosphere soil of various crop plants. A total of 22 fungi were isolated from the rhizosphere soil and screened for their ability to solubilize phosphates in PKV medium. Of these 22 rhizosphere fungi, only *Penicillium* sp. RF UOM 14 was found positive for phosphate solubilization. Similarly, there are reports on both bacterial and fungal strains exhibiting P solubilizing activity by the formation of clear halo zone (a sign of solubilization) around their colonies [16]. The results correlate with the findings of Mendes *et al*. [18], where isolates of *Aspergillus niger* FS1, *Penicillium canescens* FS23 and *Eupenicillium ludwigii* were able to solubilize all forms of P. The phosphate-solubilizing microbes showing greater solubilization (both qualitatively and quantitatively) of insoluble P under *in vitro* conditions were selected for field trials prior to production in bulk for ultimate transmission as a biofertilizer [16]. It is also suggested that phosphate solubilization, production of IAA, and other related compounds by the fungus will interact with plants as part of its colonization, leading to growth promotion, induced resistance, and modification of basal plant defense mechanisms [19-22].

Further, in the present study, conidial suspension and culture filtrate of the phosphate solubilizing fungi *Penicillium* sp. RFUOM 14 was used as seed treatment to different crop plants. In comparison with the SDW control, a significant enhancement of seed germination and vigour was noticed in phosphate solubilizing fungi *Penicillium* sp. RFUOM 14, with maximum germination of 86% and seedling vigour index score of 985 in pearl millet. Similarly, there was also significant increase in seed germination and vigor in tomato and brinjal plants also. Sudisha *et al*. [23] reported that, tomato seeds treated with phosphate solubilizing fungi like *Trichoderma*, *Penicillium*, etc., enhanced seed germination and seedling vigor. Similarly, *A. niger* and *P. italicum* solubilize tri-calcium phosphate (TCP) and improve the growth of soyabean in TCP amended soil [24]. Likewise, seeds treated with *T. harzianum* in sunflower [25] and *Penicillium chrysogenum* and *P. oxalicum* in pearl millet [13, 26] which were isolated from the rhizosphere soil offered enhanced seed germination and seedling vigour over the control.

5. CONCLUSIONS

Nowadays, PSM technology largely depends on the development and distribution of good quality inoculants to farming communities. Therefore, there is a need for extensive and consistent research efforts to identify and characterize more PSM with greater efficiency for their ultimate application under field conditions. Hence the results of the present study may contribute to the findings of other researchers, where we were able to notice enhancement in seed germination and vigor of crop plants when treated with conidial suspension and culture filtrate of phosphate solubilizing fungi *Penicillium* sp. RFUOM 14. Furthermore,

researchers need to address certain issues, like how to improve the efficacy of biofertilizers, what should be an ideal and universal delivery system, how to stabilize these microbes in soil systems, and how nutritional and root exudation aspects could be controlled in order to get maximum benefits from PSM application.

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