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RESEARCH ARTICLE

TESTING EFFICACY OF SCREENED PHOSPHATE SOLUBILISERS UNDER *IN VITRO* CONDITIONS

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ABSTRACT

Phosphate solubilising bacteria (PSB) and fungi (PSF) were enumerated by employing Pikovskaya and Modified Pikovskaya agar supplemented with Tricalcium phosphate (TCP) as insoluble phosphate source. The P solubilisers formed yellow halos around their colonies on Modified Pikovskaya agar (MPVK) using Bromophenol blue. The frequency of PSB was highest in the Kharar soil (5.1%) and the least in Model Town, Ludhiana soil (2.2%) in rhizosphere zone. The population of PSF was maximum in Kharar soil (15%) followed by Pakhowal Road, Ludhiana soil (10%) and the least in Model Town, Ludhiana soil (5.6%). The PSB reported belonged to *Bacillus* and *Micrococcus sp.* while the PSF belonged to the *Aspergillus niger, Aspergillus fumigatus* and *Penicillium* sp. The population of PSM was highest in the rhizosphere than that of non-rhizosphere. The maximum halo size on Modified Pikovskaya agar was reported in *Bacillus sp.* (5 mm). PSM were screened to observe their efficacy to solubilise P on PVK broth. *Penicilliu sp*. was found to solubilise maximum TCP (54.2 µg/ml) followed by the *Aspergillus fumigatus* (51.4 µg/ml) and the least in *Aspergillus niger* (11.2 µg/ml). The inverse trend in the rate of P solubilisation with that of final pH of broth was observed. The maximum decrease pH was reported in *Penicillium sp*. (4.5) followed by *Aspergillus fumigatus* (5.5) and *Aspergillus niger* (6.0).

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INTRODUCTION

Improving soil fertility is important for agriculture and forest production. Phosphorus (P) is one of the essential nutrients, limiting the plant growth. It is a deficient nutrient in most soils, despite of its low solubility, chemical and reversion property in soluble to insoluble P are some of the major problems. The release of fixed and poorly soluble form of P is an important aspect of increasing soil fertility (Chatli *et al.,* 2017). P also plays a very important role in carrying out all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, respiration (Khan *et al.,* 2010) and N_2 fixation in legumes. The phosphate content in average soil is all about 0.5% (w/w) but only 0.1% of the total P is available to plants because of poor solubility and its fixation in soil (Illmer and Schinner, 1995). Chemical P fertilizers impose adverse environmental impacts on overall soil health and degradation of terrestrial fresh water and marine resources (Tilman *et al.,* 2001). Phosphate solubilising microorganisms (PSM) are beneficial to solubilise inorganic phosphorus from insoluble compound (Chen *et al.,* 2006). P solubilisation ability of microorganisms is considered to be one of the most important traits associated with plants phosphate nutrition.

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Phosphate solubilisers can be used along with rock phosphate which can save about 50% of the crop requirements of phosphoric fertilizers. The use of PSB as inoculants increase P in plants. PSB can be applied in the form of fertilizers. Many different strains of these bacteria have been identified as PSB including *Pantoea agglomerans (P5), Microbacterium laevaniformans (PT)* and *Pseudomonas putida (P13).* It is also demonstrated that four bacteria synergistically solubilize phosphorus at a much faster rate than that of any single strains alone. PSB have potential to enhance phosphate inducing immobilization of metals to remediate contaminated soil. P biofertilisers have been proved to be the best eco-friendly means for crop nutrition. Although several bacterial (*Pseudomonas* and *Bacillus)* and fungal strains (*Aspergillus* and *Penicillum*) have been identified as PSM but their efficiency needs to be improved either by using genetic modification and co-inoculative techniques (Yasser *et al.,* 2014). Many fungal species which can solubilise the rock phosphates are the *Aspergillus niger, Aspergillus tubingensis, Aspergillus fumigatus, Aspergillus terrus, Aspergillus awamor, Pencillium italicum, Penicillum radicum (*Gizaw *et al.,* 2017). Moreover, the efficiency of applied P fertilizers in chemical form rarely exceeds 30% due to its fixation, either in form of Fe/Al phosphate in acidic soils (Nourish and Rosser, 1983). A number of areas of Punjab are known for their vast forest wealth. However, the forest wealth has been dwindling due to overexploitation, resulting into environmental degradation.

The urgent need is to improve forest sustainability and ecological restoration to save these precious bioresources. This can be accomplished by management of forestry practices in an integrated approach of appropriate modern technologies and traditional techniques. So, there is a need to develop biofertilisers for improving soil fertility.

MATERIALS AND METHODS

Soil Sampling: Surface soil samples (upto 30 cm depth) were collected from various soil classes viz. rhizosphere (with roots) and non-rhizosphere (roots free soil) of feeder roots of Saraca asoca (Ashoka) tree at Ludhiana (Pakhowal Road and Model Town) and Kharar (Punjab). Composite soil sample of each class were air dried and tested for microbial enumeration.

Microbial Enumeration: Total bacterial and fungal population from these soil samples was determined by serial dilution on Nutrient Agar Media (NAM) and Potato Dextrose Agar (PDA), respectively. Petriplates were incubated at 28 ± 2°C for 3 days for bacteria while at 25 ± 2° C for fungi for 5-6 days. Number of colonies were counted in the petriplates and calculated colony forming units (cfu) per gram of soil.

Isolation of Phosphate Solubilising Microorganisms: The Phosphate solubilising microorganisms were enumerated from soils samples by serial dilution on Pikovskaya (Pikovskaya, 1948) and Modified Pikovskaya agar (Gupta *et al.,* 1994). 0.4 ml dye was added to molten Pikovskaya (PVK) agar [(Modified Pikovskaya (MPVK)] to give a final concentration of 0.16 g/ml. The frequency of phosphate solubilising bacteria (PSB) and phosphate solubilising fungi (PSF) was determined as the ratio of total number of phosphate solubilising colonies isolated on Pikovskaya (PVK), Modified Pikovskaya (MPVK) agar to total number of colonies of bacteria and fungi obtained on NAM and PDA, respectively using the same soil dilution and expressed as percentage. Characterization of bacterial isolates was done on the basis of cultural, morphological and biochemical characteristics as described by Bergey's Manual (1984). Various tests were employed following (Kannan *et al.,* 2002). Identification of fungal isolates was done on the basis of various cultural and microscopic characteristics (Gilman, 1957). The isolates of bacteria and fungi forming clear zone on Pikovskaya agar and clear yellow halos on Modified Pikovskaya agar (MPVK) were transferred and purified on NAM and PDA, respectively.

Determination of Halo Size: A pin point of inoculation of bacterial and fungal culture was done on respective media viz. PVK and MPVK agar with tricalcium phosphate (TCP) as insoluble inorganic phosphate source. The plates were incubated at 28 ± 1℃ for 5 days for bacterial and 7 days for fungal culture. The microbial solubilisation of phosphate exhibited with clear zone formed around the colony and its size was measured in mm. The halo size around the colony was calculated. The experiment was carried out in triplicates.

Quantitative estimation of P solubilisation in culture broth: 10^6 bacterial cells and 30×10^5 fungal spores per ml were inoculated with 100 ml PVK broth for 5 days for bacteria and 6 days for fungi under shake at 250 rpm. Uninoculated broth served as control. The solubilised P was determined in clear filtrate using Ascorbic acid method (Watanobe and Olsen, 1965). Then the intensity of blue color was measured on spectrophotometer at 730 nm and the quantity of solubilised P was expressed as µg/ml. The final pH of culture filtrate was also determined.

RESULTS AND DISCUSSION

Enumeration and identifications of microorganisms (Bacteria and Fungi): All the collected soil samples were used to enumerate bacteria and fungi including beneficiary microbes' viz. PSM to determine their inter-relationship in various zones of *Saraca asoca* (Ashoka tree) in Punjab. The rhizosphere microbes behaved differently from non rhizosphere both in terms of number and type of microorganisms. The population count of microbes was more in rhizosphere than that of non-rhizosphere. So, rhizosphere showed a positive influence of microbial population. This may be due to the production of growth promoting substances by the root of *Saraca asoca* tree resulting in increasing in competitive ability of the beneficiary microbes. The zone also results in more survival rate of microbes. The results were supported by the finding of Brandy (2001) who also reported the higher population of microbes in rhizosphere. Our results are also in corrobation with the results of Sharma *et al.,* 2013; Khan *et al.,* 2007; Baliah *et al.,* 2016. The highest bacterial community in rhizosphere was recorded in Model Town, Ludhiana (268×10^4/g soil) followed by Pakhowal Road, Ludhiana (60×10^4/g soil) and the least in Kharar (58×10^4/g soil). In non-rhizosphere, highest population also reported in Model Town, Ludhiana (124×10^4/g soil) followed by Pakhowal Road, Ludhiana (37×10^4/g soil) while the least in Kharar (33×10^4/g soil) (Table 1). Thus, the population of total bacteria in rhizosphere was reported to be highest as compared as to non-rhizosphere. Majority of bacterial isolates belonged to *Bacillus and Micrococcus* species. The total fungal population densities were also decreased in non-rhizosphere (10×10^4/g of soil) while the highest fungal population was observed in rhizosphere (53×10^4/g of soils). In rhizosphere the highest fungal population was reported in Model Town, Ludhiana (53×10^4/g of soil) followed by Pakhowal Road, Ludhiana (30×10^4/g of soil) while the least in Kharar (20×10^4/g of soil). In non-rhizosphere highest population of fungi was reported in Model Town, Ludhiana (25×10^4/g of soil) followed by Pakhowal Road, Ludhiana (20×10^4/g of soil) while the least in Kharar (10×10^4/g of soil) (Table 2). Hence, the population of total fungi was also more in rhizosphere than that of non-rhizosphere. The majority of fungi were *Aspergillus niger, Aspergillus fumigatus* and *Penicillum sp.* Antagonistic properties were also reported in case of certain bacteria. The antagonistic action can be due to production of antibiotics, hormone like substances competition for nutrients and colonization sites etc. These studies show that microbial antagonist can be used in agricultural practice for improvement of soil.

Enumeration and identification of phosphate solubilising microorganisms (PSM): Phosphate solubilising bacteria (PSB) and phosphate solublising fungi (PSF) associated with *Saraca asoca* in soils from rhizosphere and non-rhizosphere was isolated. Their population count was more in rhizosphere than non-rhizosphere sites. The phosphate solubilisation was also higher in the rhizosphere. The positive rhizospheric effect of perennial plants on microbial activity has been widely reported (Gizaw *et al.,* 2017). The limited information was available about influence of rhizospheric zone on the efficiency of phosphate solublising microorganisms (Rovira, 1991; Sundara *et al.,* 2002).

Table 1. Phosphate Solubilising Bacteria (PSB) from the natural habitat of *Saraca asoca* (Ashoka tree) from Punjab

Area		Soil sample	Mean plate count (×10^4/g Soil) Total bacterial count	Phosphate Solubilising Bacteria (PSB)		Percentage of PSB	
Punjab	Model Town, Ludhiana		NAM	PVK	MPVK	PVK	MPVK
		R	268	4	6	1.4	2.23
		NR	124	1	2	0.8	1.61
	Pakhowal Road, Ludhiana	R	60	2	3	3.3	5
		NR	37	1	1	2.7	2.7
	Kharar	R	58	2	3	3.4	5.1
		NR	33	1	1	3.0	3.0

NAM – Nutrient Agar Medium; PVK – Pikovskaya Agar Medium; MPVK – Modified Pikovskaya Agar Medium

Table 2. Phosphate Solubilising Fungi (PSF) from the natural habitat of *Saraca asoca* (Ashoka tree) from Punjab

Area		Soil Sample	Mean Plate count (× 10^4)/g soil Total count fungal	Phosphate Solubilising Fungi (PSF)		Percentage of PSF	
Punjab	Model Town, Ludhiana		PDA	PVK	MPVK	PVK	MPVK
		R	53	2	3	3.7	5.6
		NR	25	Nil	Nil	Nil	Nil
	Pakhowal Road, Ludhiana	R	30	2	3	6.6	10
		NR	20	1	1	5	5
	Kharar	R	20	2	3	10	15
		NR	10	Nil	1	Nil	10

NAM – Nutrient Agar Medium; PVK – Pikovskaya Agar Medium; MPVK – Modified Pikovskaya Agar Medium

Table 3. Tricalcium Phosphate (TCP) solubilisation of bacterial isolates in Pikovskaya and Modified Pikovskaya Agar medium

Bacteria	Treatment Agar [Halo size (mm)]	
	Pikovskaya	Modified Pikovaskaya
EBP1(*Bacillus)*	2	5
EBP2(*Micrococcus)*	1	2
EBP3(*Nonsporulating sterile)*	1	3

Table 4. Tricalcium Phosphate (TCP) solubilisation by fungal isolates in Pikovskaya and Modified Pikovskaya Agar medium

Fungi	Treatment Agar [Halo size (mm)]	
	Pikovskaya	Modified Pikovaskaya
EFP1(*Aspergillus niger)*	8	15
EFP2(*Aspergillus fumigatus)*	16	18
EFP3 (*Penicillium)*	7	9
EFP4(*Nonsporulating sterile)*	15	15

Table 5. Tricalcium Phosphate (TCP) solubilisation by phosphate solubilising microbes and final pH of Pikovskaya broth

Microorganism	PVK broth (µg/ml P solubilised)	Final pH of broth
Bacillus sp.	49.4	5.8
Aspergillus niger	11.2	6.0
Aspergillus fumigatus	51.4	5.5
Penicillum sp.	54.2	4.5

Initial pH of broth = 6.8

On the Pikovskaya agar the community of PSB in rhizosphere was higher in Kharar (3.4%) followed by Pakhowal Road, Ludhiana (3.3%) and the least in the Model Town, Ludhiana (1.4%). In the non-rhizosphere community was also reported to be highest Kharar (3%) followed by Pakhowal Road, Ludhiana (2.7%) and the least in Model Town, Ludhiana (0.8%). The population of P-solubilisers was higher in Kharar on Modified Pikovskaya agar than that of Pikovskaya agar or least in the Model Town, Ludhiana. The highest PSB count in rhizosphere was recorded in Kharar on Modified Pikovskaya agar was (5.1%) followed by Pakhowal Road, Ludhiana (5%) and the least in Model Town, Ludhiana (2.23%). In non-rhizosphere population was also reported to be higher in Kharar (3%) followed by the Pakhowal Road, Ludhiana (2.7%) and the least in Model Town, Ludhiana (1.16%) (Table1).

On the Pikovskaya agar total fungal count in rhizosphere was higher in Kharar (10%) followed by the Pakhowal Road, Ludhiana (6.6%) and the least in Model Town, Ludhiana (3.7%). In non-rhizosphere the PSF count was found only Pakhowal Road, Ludhiana (5%). The highest PSF count in rhizosphere was recorded in Kharar on Modified Pikovskaya agar (15%) followed by the Pakhowal Road, Ludhiana (10%) and the least in Model Town, Ludhiana (5.6%). In non-rhizosphere on Modified Pikovskaya Agar (MPVK) the higher fungal count was in Kharar (10%) followed by the Pakhowal Road, Ludhiana (5%) and the Nil in Model Town, Ludhiana. The low total viable count of bacteria and fungi as well as the low viable cont of PSB and PSF in the soil sample of Model Town, Ludhiana were possible due to temprature and moisture or coupled fertility level (Table 2).

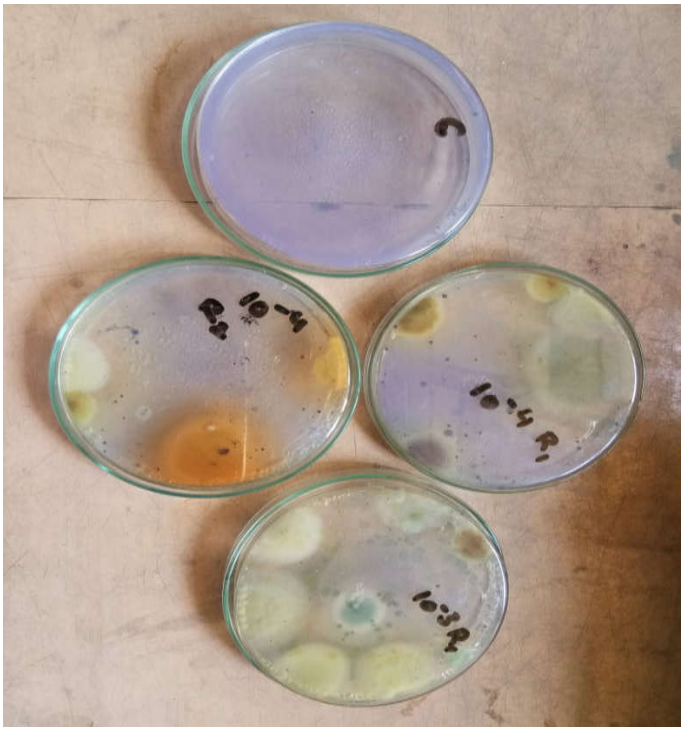

Fig. 1 Phosphate solubilising microbes on MPVK

Fig. 2 Tricalcium phosphate solubilisation on PVK broth

The highest population of PSM was reported on Modified Pikovskaya (MPVK) than that of Pikovskaya (PVK) (Fig. 1). It may be due to the binding of basic dye Bromophenol blue to the genes of organisms which caused their activation for the production of enzyme phosphatase resulting in increase in P solubilisation due to acidification of media (Table 1, Table 2). Highest solubilised Tricalcium phosphates in PVK broth medium by *Penicillum sp.* (54.2 µg/ml) followed by *Aspergillus fumigatus* (51.4 µg/ml) and *Bacillus sp*. (49.4 µg/ml) and least solubilised by the *Aspergillus niger* (11.2 µg/ml) (Table 5) (Fig. 2). Majority of PSB isolates belonged to the *Micrococcus* and *Bacillus*. Majority of PSF isolates were *Aspergillus niger, Aspergillus fumigatus and Penicillum.* Peclzar (1993) also reported that the microorganism proliferated in rhizosphere better than anywhere else as they obtain their nutrition from root exudates, plants mucigel and root lysates.

Conclusion

The phosphate solubilising microorganisms act as alternative to chemical fertilizers. Soluble phosphorus is essential for the crop development and output. The phosphate solubilising efficacy of the isolated species could be used to solubilise higher amount of phosphates found in the soils of Punjab. The results indicate that for maximum benefits there is a need to develop plants growth promoting microbial formulations.

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