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In vitro Solubilization of Inorganic Phosphate by Phosphate Solubilizing Fungi Isolated from Tea Agroecosystem Soil of Barak Valley, Southern Assam

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Abstract: Tea {*Camellia sinensis* (L.) O' Kuntze} is an economically important major plantation crop of India. Phosphate is an important nutrient for the establishment of young tea plants. The problem of phosphorus nutrition in tea plants is complex because only 15-25 % of added phosphorus is utilized by the plants and remaining of it gets fixed in the soil. There are a variety of microorganisms in soil which can solubilize insoluble phosphates and mineralize organic phosphorus. Among the microorganisms, the fungi are the most efficient phosphate solubilizing microorganisms. Microbial solubilization of fixed mineral phosphates in the soil is an important process in natural ecosystems and in agricultural soils. In the present work, seven most efficient phosphate solubilizing fungi were isolated from tea agroecosystem soil from Barak Valley, Southern Assam. *In vitro*, potential of the P-solubilizing activity by these PSMs were observed. These fungal strains developed halozones on PVK agar, which is an indicator of phosphate solubilization. The solubilizing efficiency of these fungal strains in liquid culture was also determined spectrophotometrically.

Key words: {*Camellia sinensis* (L.) O' Kuntze} · Psms · P-Solubilizing Activity · Halozones · Soil Fungi

INTRODUCTION

Microbial solubilization of less soluble mineral phosphates in soil is an important process in natural ecosystems and in agricultural soils. In spite the soil usually contain a high amount of total phosphorus, its availability to plants is very slow and it is often a limiting factor for the plant growth. In addition to traditional methods of mineral phosphate fertilization, microbial P- solubilization may increase the availability of phosphates in arable soils. Besides, phosphorus applied to soil as mineral phosphate fertilizers is transformed (From 70- 90%) to slowly available compounds and the effectivity of its uptake by plants is relatively low [1, 2]. Microbial P- solubilization can, therefore improve the effectivity of mineral P fertilization.

Phosphorous is second only to nitrogen among the mineral nutrients, most commonly limiting the growth of the crop i.e. tea. Phosphorous is an essential element for plant development and growth making up about 0.2% of plant dry weight. The role of microorganisms in solubilizing inorganic phosphates in soil and making them available to plants is well known [3]. Phosphorous is one of the major essential plant nutrients. After its application, a large proportion of phosphatic fertilizer is converted to insoluble form. However, the availability of phosphorus in the soluble state is of highly agronomic value. It was reported that many soil fungi and bacteria can solubilize inorganic phosphate [4-6]. [7] found fungi more active in solubilizing phosphate than bacteria. Omar [8] tested 36 fungal species on agar plates. Most of them were non solubilizers. *Aspergillus niger* and *Penicillium citrinum* caused a marked drop in pH of the liquid culture media and solubilized considerable amounts of phosphate.

Solubilization of rock phosphate or other insoluble forms of elemental P by fungi and other microrganisms has been studied earlier. In agreement with the present results, species of *Aspergillus, Penicillium, Curvularia* and yeasts have been widely reported for solubilizing various forms of inorganic phosphate [4, 9-11]. Kumar and

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Narula [12] evaluated different mutant phosphate solubilizing strains of *Azotobacter chroococcum* for their ability to solubilize insoluble phosphate and indole acetic acid (IAA) production on the basis of clear zone produced on Pikovskaya's and Jensen's media. The appearance of a clear halo around the colony of *Eupenicillium paravum* indicated phosphate solubilization by the fungus [13, 14]. Kim *et al*. [15] indicated that the population of phosphate solubilizing bacteria depends on cultural activities and different soil properties (Physical and chemical properties, organic matter and soil phosphorus content). It has been suggested that in acidic soil with low availability of phosphorus, rock phosphates could be used through the action of microbial solubilisation [16].

In the pesent work our aim of the present work was to isolate and identify the microbial species to measure their phosphate solubilizing potential *in vitro* so that they can be utilized for the benefit of the tea industry at large.

MATERIALS AND METHODS

Soils samples were collected from the hot and cold slope of old tea plantation area as well as from hot and cold slope of young tea plantation areas of Rosekandy Tea Estate. After removal of the debris and litter at 0-15cm. depth the soil samples were collected. They were analyzed to isolate PSMs and to observe their potential to solubilize Tricalcium phosphate.

Soil Dilution Plate Method: Timonin, 1940 10gm of the soil was taken in a 250ml conical flask containing 100ml sterile water. This stock solution was thoroughly hand shaken for about 10-15 minute. The dilution in the flask was treated as 1: 100.Subsequently, dilution i, e 1:10000, was prepared for the isolation of fungi 1ml of the prepared solution as described above were inoculated in the respective petidishes. The respective media put in the petridishes (10ml each approx) and shaken gently for few seconds. While pouring the media in petridishes, care was taken to keep the cover of the petidishes close to prevent contamination. The plates for fungus were incubated for 5-7 days at 25±2°C After the incubation period the growth of fungi was observed [17].

Sceening for Phosphate Solubilizing Micorganisms (Psms): Samples collected from tea agroecosystem were screened for phosphate solubilizing fungal population with the help of the method of Pikovskaya. The tricalcium phosphate (TCP) was the sole phosphorus source and was used selectively to screen each fungal isolate for its ability to release inorganic phosphate. The pure cultures of the fungal colonies showing clear zone on Pikovskaya's agar medium were identified, sub-cultured and stored for further use.

Solubilization Index (SI): Sterilized Pikovskaya's media was poured into sterilized Petri dishes, after solidification of the media; a pin point inoculation of Petri dishes was made on the dishes under aseptic conditions. The dishes were incubated at 28°C for 7 days. Then the ability of PSM to solubilize the insoluble phosphate was studied by following the of solubilization index: the ratio of the total diameter (colony + halozone) and the colony diameter [18]. The experiment was performed in triplicate.

$$SI=\frac{\text{Colony diameter + halozone diameter}}{\text{Colony diameter}}$$

pH Change: 1 ml of the three day old culture in sterile distilled water was added to sterile 100ml Pikovskaya's broth (PB) medium in 250ml conical flask and kept for (7x4) days. Sterile uninoculated medium served as control. Initial pH and change in pH was noted at an interval of seven days with the help of digital pH meter.

Test for Available P: Broth P was determined by Ascorbic acid [19]. One ml of broth sample extract was taken in 50 ml conical flask and 9ml of distilled water + 2.5 ml of freshly prepared coloured reagent [12 gm ammonium molybdate + 250 ml distilled water and 0.29808 mg antimony potassium tartarate in 1000 ml of 5N H_2SO_4 (148 ml conc. H_2SO_4/ l. Both the solutions were mixed and the volume was raised upto 2l.140 ml. This mixture was added to 0.74 g ascorbic acid and stirred gently. The optical density of the blue colour developed after 15min was measured at 880 nm by spectrophotometer and the concentration of available P was determined.

RESULTS AND DISCUSSIONS

The fresh soil samples collected were used for isolation of various soil microorganisms by serial dilution plate method. These were used for various experimental purposes. The soil samples collected from tea agroecosystem are acidic in nature. The acidity may be due to the leaching of the base materials or due to the rapid decomposition of the organic matter.

However, it can be noted that acidic soil (pH 4.5-5.5) favours the growing of tea and the area from which soil samples were collected is suppose to be good for growing tea. The phosphate solubilizing fungi were present in all the soil samples. However, they were found to differ both in efficiency as observed from the clear zone diameter around their colonies in the phosphate amended Petri dishes containing PDA. The isolates were identified on the basis of their cultural, morphological characters with the help of the available references i.e.A Manual of soil fungi-by. Gilman *et al.* [20], Illustrated Genera of Imperfect Fungi- by Barnet *et al*.[21], Monographic contribution on *Trichoderma*- by Nagamani *et al.* [22], The Genus *Aspergillus*- by Raper and Fennel [23].Subsequently, the cultures were sent to IARI, Delhi for the confirmation of their identification.

When grown in culture media was supplemented with tricalcium phosphate, all the isolates produced halozone around the colonies, indicating the solubilization of phosphate source used. Phosphate solubilizing microbes were seeing by the formation of clear halos around their colonies. The halo is produced due to the solubilization of insoluble phosphates, which in turn is mediated due to the production of organic acid in the surrounding medium [24]. Solubilization index based on colony diameter and halozone for each PSM isolates were presented in Table 1. Results showed that among PSM, *Trichoderma asperellum* was most efficient phosphate solubilizer on Pikovskaya's agar (PA), Pikovskaya [25] plates with SI=1.87, followed by *T. harzianum*, *T. viride, T. citrinoviride* with a SI of 1.79, 1.68 and 1.62 respectively, while, *Aspergillus niger* and *A. flavus* showed a SI value of 1.58 and 1.52 respectively and the least was noted by *Penicillium funiculosum* with a SI value of 1.41. Generally, halozone increased with increase in colony diameter. Studies on agar plates revealed that phosphate solubilizing fungi formed clear zones by solubilizing suspended tricalcium phosphate. Most of the studied fungi produced larger halozones with time. (Plate 1). These results are in accordance to [26, 27]. Similar results were also reported by [12, 18, 28, 29]. Most of the PSM strains lost their ability to form halozone on PA medium on repeated subculturing. This result is in accordance to Kucey and Leggett [30] and Illmer and Schinner [31].

Table 1: Solubilization index (SI) of selected strains of phosphate solubilizing fungi isolated from tea agroecosystem soil.

Strains	Solubilization Index Seven Days time of inoculatio
Aspergillus niger	1.58
Aspergillus flavus (8922.12)	1.52
Penicillium funiculosum (8921.12)	1.41
Trichoderma harzianum	1.79
Trichoderma citrinoviride (8923.12)	1.62
Trichoderma asperellum (8920.12)	1.87
Trichoderma viride	1.68
Control	0.00
LSD (5%)	0.793*
LSD (1%)	1.201*

(Each value is based on the mean of 3 replicates)

The results of tri-calcium phosphate solubilization by the selected isolates has been shown in Table 2. Based on the results it may be concluded that *Aspergillus niger* and *Trchoderma asperellum* were the most efficient strains among the tested fungi, followed by *T. citrinoviride,* although all the fungal strains were found to be good solubilizers. Solubilized P by all the studied PSM strains was higher than uninoculated control.

Table 2: Phosphate solubilizing efficiency of fungal strains isolated from tea agroecosystem soil

	Amount of P (mg 50/l)			
Fungal strains	7 days	14 days	21 days	28 days
Control	25.3	25.3	25.3	25.3
Aspergillus niger	96.8	121.3	196	340.5
Aspergillus flavus (8922.12)	80.7	91.1	117.2	272.1
Penicillium funiculosum (8921.12)	64	90	130	216.3
Trichoderma harzianum	77.2	81.4	106	236.8
Trichoderma citrinoviride (8923.12)	74.3	86.1	113.7	300.8
Trichoderma asperellum (8920.12)	84.1	94.9	157	328.8
Trichoderma viride	71.03	90.6	109	204
LSD (5%)	8.887	10.047	13.498	19.397
LSD (1%)	13.124	14.837	19.934	28.645

(Each value is based on the mean of 3 replicates)

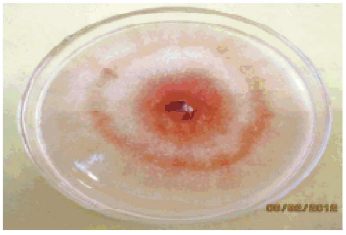
Table 3: Drop in pH by selected strains of phosphate solubilizing fungi isolated from tea agroecosystem soil.

	Drop of pH drop during twenty eight days of incubation.			
Fungal strains	Day 7	Day 14	Day 21	Day 28
Control	7.09	7.09	7.09	7.09
Aspergillus niger	5.99	4	3.43	3.20
Aspergillus flavus (8922.12)	6.61	5.19	4.74	3.33
Penicillium funiculosum (8921.12)	5.58	4.91	3.72	3.30
Trichoderma harzianum	6.16	5.08	3.75	3.33
Trichoderma citrinoviride (8923.12)	6.06	4.82	3.95	3.25
Trichoderma asperellum (8920.12)	6.05	4.58	3.72	3.43
Trichoderma viride	6.33	4.91	4.60	4.30
LSD (5%)	1.303	1.829	2.109	2.236
LSD (1%)	1.924	2.701	3.114	3.302

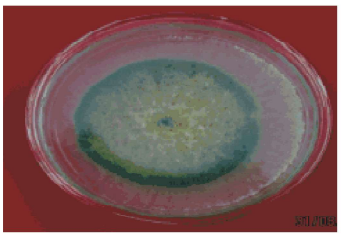
(Each value is based on the mean of 3 replicates)

A *Trichoderma citrinoviride*

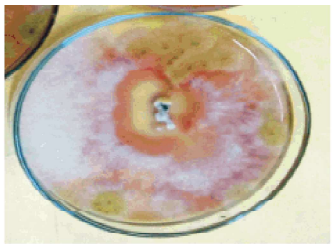
B *Trichoderma asperellum*



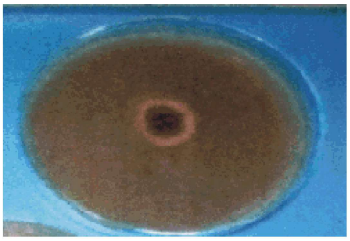
C *Trichoderma viride*



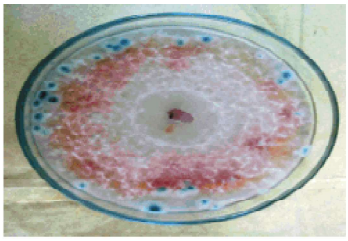
D *Trichoderma harzianum*



E *Aspergillus flavus*



F *Aspergillus niger*



G *Penicillium funiculosum*

Plate 1: Plate ABCDEFG showing halozone produced in the ticalcium medium of the inoculated PSMs.

These results are in accordance with Alam Sadia *et al*. [32]. On the 7th day of incubation, *A. niger* showed highest solubilizing efficiency as compared to other strains and control. The same fungal species was also found to solubilize the tricalcium phosphate on the 14th, 21st and 28th day of incubation. The solubilization increased steadily up to 28 days of incubation where the maximum amount of soluble phosphorous was released.

It was interesting to observe that there was a drastic drop in the pH of the medium inoculated with all PSM isolates. While the pH remains the same in the uninoculated control throughout the 28 days of incubation/ observation. Drop in pH by PSM ranged from 4.30 to 3.2 at the end of the incubation period. These results are in accordance with Alam Sadia *et al.* [32]. There was a perfect inverse relation with the amounts of tri-calcium solubilized and the pH of the medium inoculated with PSM cultures. The highest amount of tricalcium phosphate solubilization was achieved by *Aspergillus niger* with the lowest pH of 3.20. This is in accordance with Keneni *et al.* [33]. Acid production and reduction of the pH of the medium is one of the mechanisms by which soluble phosphorus is released by PSB [34].

CONCLUSION

Results obtained with the present study shows that *Aspergillus niger*, *Trichoderma asperellum* (8920.12) and *Trichoderma citrinoviride* (8923.12) are the most efficient fungal strains as far as their phosphate solubilizing potential. Further study are being done with these fungal strains to observe their concerned potential to increase the productivity of tea plants under the nursery and field conditions.

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