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Phosphate Solubilizing Potentiality of Fungi in Rhizophore of *Camellia sinensis* (L.) O. Kuntze

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Abstract

The purpose of the present study was to isolate fungi from tea rhizosphere and to assess their phosphate solubilisation ability. Fungal species were first isolated on RBA (Rose Bengal Agar) plates following serial dilution method. Colonies formed were then transferred to PVK (Pikovskaya) plates and broths to isolate possible phosphate solubilisers. Factors like SI (Solubilisation index) in plates, final pH and quantity of phosphate solubilsed in broths by these isolates were recorded. Relation between quantuity of phosphate solubilised and final pH in broth were recorded. Isolated *Aspergillus* sp. and *Penicillium* sp. showed solubilisation index ranging from 1.7 to 1.2. Maximum phosphate solubilisation was recorded (2.79 mg P_2O_5/ml) in broth of *Aspergillus* sp.1 after 15 days. Highest drop in pH was recorded from broth of Aspergillus sp.2 (7.0-3.9). Pearson's correlation between final pH of broth and solubilisation of insoluble phosphates by isolates showed significant (P>0.01) negative relation (r = -.972**). Strains of phosphate solubilizing *Aspergillus* and *Penicillium* isolated in the present study represent potential bio-inoculum as biofertilizers for economic crop like tea.

Key words: Bio-fertilizers, Fungi, Phosphate.

Introduction

Phosphorus, a nutrient next only to nitrogen in plant requirement plays key role in plant development and crop yield. Sources of phosphorus in soil include chemical fertilizers, plants and animals residue, and native phosphorus compounds present in organic as well as in inorganic form in the soil (Rao, 1982). Plants require phosphorus for their physiological activities such as cell division, photosynthesis, root system development and carbohydrate utilization (Sharma *etal*., 2011). Approximately 95-99% of soil phosphorus is associated with different compounds in soil (Son *etal*., 2006) making it the least available element for plant usage. Therefore phosphorus fertilizers are being applied continuously in areas harbouring economic crop like tea for enhancing crop productivity leading to disruption in environmental cycles. To enhance available phosphorus level, fertilizers like rock

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phosphate are being applied in agricultural areas (Zapata and Axmann, 1995). Despite addition of fertilizers, only limited amount of phosphorus are available for plant usage (Abd, 1994) due to its association with different compounds in soil like aluminium, calcium and iron (Son *etal*., 2006) and also applied phosphorus are readily accumulated in soil resulting in environmental pollution (Ju *etal*., 2007). Groups of microbes in soil called phosphate solubilizing microbes are able to solubilize insoluble phosphate through organic acids synthesis (Nopparat *etal*., 2007), chelation, acidification, ion-exchange reactions and polymeric substance formation (Delvasto, 2011) thereby, supplementing phosphorus to plants for their growth and productivity. Populations of phosphate solubilizers vary depending upon crop plants and these differences may be due to soil factors and enzyme activities in their growth environment (Ponmurugan and Gopi, 2006). Efficiency of phosphate solubilization by soil microbes depend upon soil environment which is the sole source of soil nutrients phosphate, carbon and nitrogen. Factors such as microbial population size, pH and enzyme activities in soil also to a great extent determine microbial phosphate solubilization (Varsha *etal*., 2010). Plants associating with phosphate solubilizers in soil contain more phosphate source use efficiencies as a result of their combination that increase agricultural production sustainability in an area by reusing phosphate residues resulting from microbial metabolism (Sperber, 1958). Soil microbes thus, play critical role in soil phosphorus cycle and they provide many advantages over synthetic fertilizers. Microbes possessing ability to solubilize phosphate include genera belonging to bacteria, fungi and actinomycetes (Kucey, 1983; Waksman, 1992) however fungi possess greater ability in solubilizing rock phosphate (Nahas, 1996). Among phosphate solubilizer fungi, *Aspergillus* and *Penicillium* are the dominant genera in rhizospheric soil (Wakelin *etal*., 2004). Inoculating such microbes with natural phosphate bearing materials such as rock phosphates could therefore, improve soil physiochemical and biological properties as well as enhance crop production (Elias *etal*., 2016). Better understanding of the underlying mechanism of phosphate solubilization by microbes and identification of superior strains of phosphate solubilizing microbes can be an alternative environment friendly solution to help reduce or replace dependence on chemical fertilizers while still promoting growth and yield of economic crop like tea.

Methods

Sample Collection

The rhizospheric soil samples were collected in the month of April 2016 in tea (*Camellia sinensis*) garden with geographical coordinates N 26°17'30.6 E 094°28'29.2 under Mokokchung district, Nagaland. Soils adhering to roots of *Camelliasinensis* were collected randomly from five sites. Samples were transferred to laboratory under sterile condition and stored at 4°C for analysis.

Isolation of Fungal Isolates

Fungal strains were isolated following serial dilution method (Waksman, 1922) on Rose Bengal agar (Martin, 1950) plates supplemented with streptomycin to inhibit bacterial growth. 10 grams of soil samples werere suspended in 90 ml of autoclaved distilled water. After performing serial dilution upto 10^{-6}, 0.1 ml each of 10^{-4}, 10^{-5}, 10^{-6} were platted on the medium in triplicates by spread plate technique and incubated at 27 ± 2°C in the dark for 5 days. Colonies formed on plates after incubation was streaked and re-streaked

on fresh RBA (Rose Bengal agar) plates to obtain pure cultures.

Screening for Phosphate Solubilizing Fungi

All fungal isolates from RBA were screened for their phosphate solubilizing ability. The isolates were spot inoculated in triplicates on Pikovskaya agar plates (Pikovskaya, 1948) supplemented with 0.5% tri-calcium phosphate and incubated at 30°C for 5 days. Uninoculated plate was also incubated at the same temperature to serve as control. Fungi forming halo zones after 5 days incubation were transferred to RBA slants and stored at 4°C for identification. Positive isolates were identified basing on their colony morphologies such as surface appearance, texture and colony colours and microscopic studies under compound microscope using lactophenol cotton blue staining method. Relevant fungal identification manuals following Domsch (1980) and Gilman (1956) were used for identification of the isolates upto genus level.

Solubilization Index

Isolates forming clear halozones after 5 days of incubation were taken as potential phosphate solubilizers. Phosphate SI (solubilization index) was calculated using the formula (Edi-Premono *etal*., 1996):

SI (Solubilization index)

$$= \frac{\text{Colony diameter + clearing zone}}{\text{Colony diameter}}$$

Quantitative Estimation of Phosphate Solubilization

Spore suspensions were prepared in PVK broth by inoculating fungal spores with sterile loop and kept in shaker for 15 days at 27 $\pm$ 2°C. Cultures were then filtered and supernatants were analyzed to record concentration of available phosphorus and pH drop in broths. Available phosphorus was determined spectrophotometrically by Olsen's method (Rai, 2002) using ELICO double beams SL210.

Data Analysis

Standard deviations and standard errors of standard deviations were done using Microsoft excel 2007 and Pearson's correlation between phosphate solubilized by isolates and final pH of broths were calculated using spss 16.0.

Results and Discussions

After 5 days of incubation, distinct fungal colonies were formed on RBA plates (Fig.1). Morphological characteristics of isolates showed 12 different fungal colonies which were subjected to further purification by re-streaking on fresh RBA plates. However, when transferred to PVK plates only four isolates were found to be capable of solubilizing tri-calcium phosphate. Clear halo zones were formed around the plates of positive isolates (Fig.2) which is an indication of phosphate solubilization. These isolates were identified as species belonging to *Aspergillus* and *Penicillium* and were named as *Aspergillus* sp.1, *Aspergillus* sp.2, *Penicillium* sp.1, *Penicillium* sp.2 (Fig.3). Colony colours of *Aspergillus* species in pure culture plates of RBA appeared black and yellowish green on front side of petriplates but on reverse colours were yellowish with brown spots in the centre and brownish white. In both the species, hyphae were septate with round conidia arranged in chains. Species of *Penicillium* on front side of pure culture plates of RBA were dull green and dark green and on reversing the plates, colour of colonies were light black. Conidiophores were borne on septate hyphae and phialides gave brush like appearance.

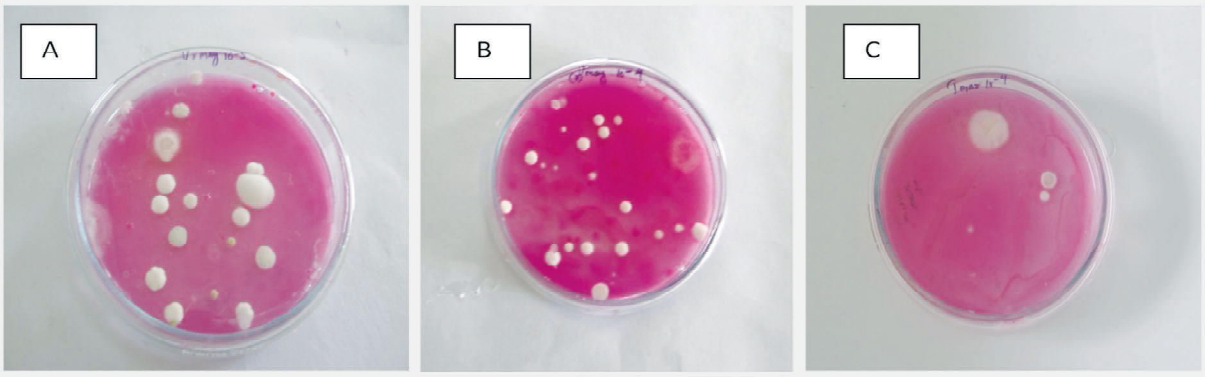

Fig. 1: (A-C) Colonies formed in Rose Bengal agar plates.

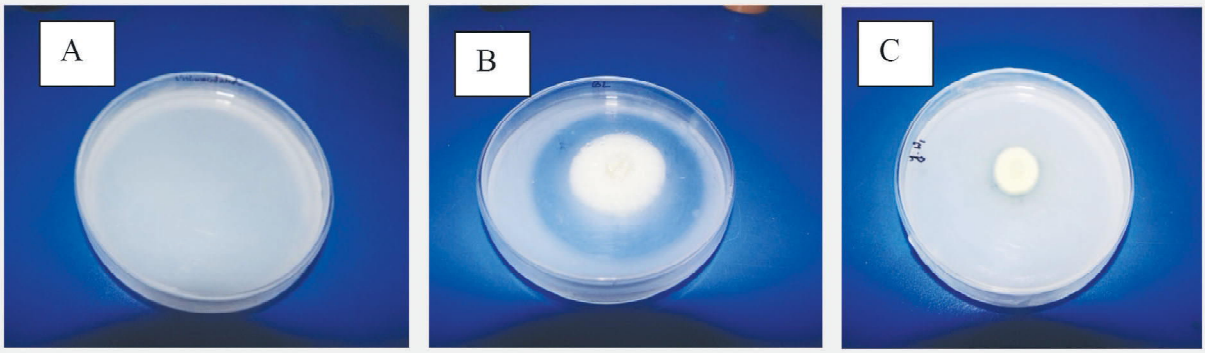

Fig. 2: (A) Uninoculated Pikovskaya plate, (B-C) formation of halo zone around colonies of Aspergillus sp. and Penicillium sp. in Pikovskaya agar plates.

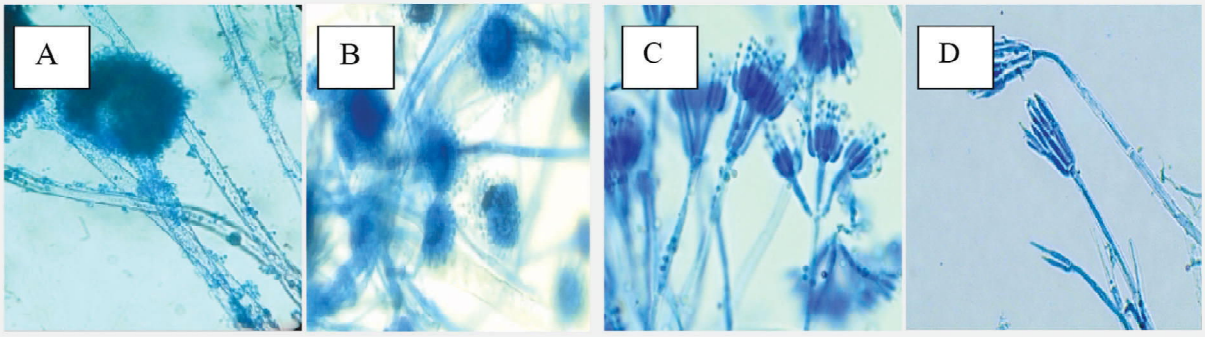

Fig.3: Microscopic photos of isolates at 100x magnification. (A) Aspergillus sp.1,(B) Aspergillus sp.2, (C) Penicillium sp.1, (D) Penicillium sp.2.

All the positive isolates showed different SI ranging from 1.2 to 1.7 cm (Fig.4). Colony diameters of *Aspergillus* species were observed to be larger than those of *Penicillium* species and clearing zones were also found to be larger in plates of *Aspergillus* species. Highest SI was observed in plates inoculated with *Aspergillus* sp.1 and the lowest SI was from plates of *Penicillium* sp.1. Higher phosphate solubilization efficiency of *Aspergillus* species compared to than *Penicillium* was also reported by El-Azouni (2008).

In the present study isolates when grown in PVK broths were found to release high amount of phosphates. Amount of phosphate solubilized in broths were as follows *Aspergillus* sp.1- 2.79 mg P_2O_5/ml, *Aspergillus* sp.2 – 2.64 mg P_2O_5/ml, *Penicillium* sp.1- 2.68 mg P_2O_5/ml, *Penicillium* sp.2-2.49 mg P_2O_5/ml (Fig.5). Phosphate solubilization by *Aspergillus* and *Penicillium* have been largely attributed to their ability to secrete organic acids such as formic acid, acetic acid, propionic acid, lactic acid, glycolic acid, fumaric acid and succinic acid (Rashid et al., 2004). Species of *Aspergillus* and *Penicillium* as potential phosphate solubilizing fungi have been reported from rhizospheric soil samples by others as well (Onyia *etal*., 2015). *Aspergillus* sp. 1 was able to release highest quantity of phosphate while the lowest quantity was released in broth inoculated with *Penicillium* sp.2. This may be due to better P- pool utilization by *Aspergillus* sp.1 (Phukan *etal*., 2012).

According to previous workers (Kucey, 1983; He and Zhu, 1988; Pradhan and Sukla, 2005; Kapri and Tewari, 2010) phosphate solubilization by microbes in broth occurs due to release of diffusible organic acids which in turn lowers broth pH. Lowering of broth pH with phosphate solubilization was also observed in the present work. pH of broths with inoculums dropped significantly from initial pH 7 to 3.9, 4.2, 4.4, 4.5 with highest pH drop in broth of *Aspergillus* sp.2 (Fig.6). Pearson's correlation between final ph of broth and phosphate solubilized by isolates showed significant ($p > 0.01$) negative relation (-.972**) (Table.1) indicating that drop in pH alone cannot determine quantity of phosphates solubilized in broth. This finding is in agreement with previous works (Kucey, 1983; Nopparat et al., 2007; Marra *etal*., 2015).

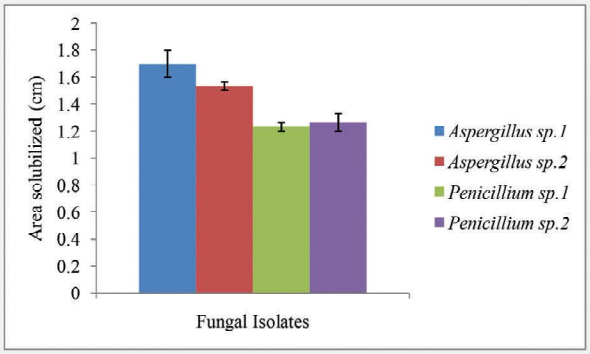

Fig. 4: Formation of clearing zones by fungal isolates. Error bars represent standard error of standard deviation.

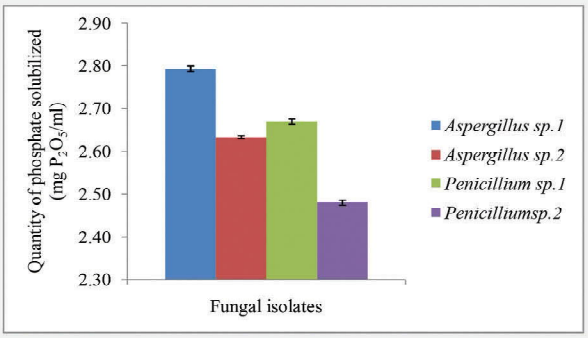

Fig.5: Amount of phosphate solubilized by fungal isolates in broth. Error bars represent standard error of standard deviation.

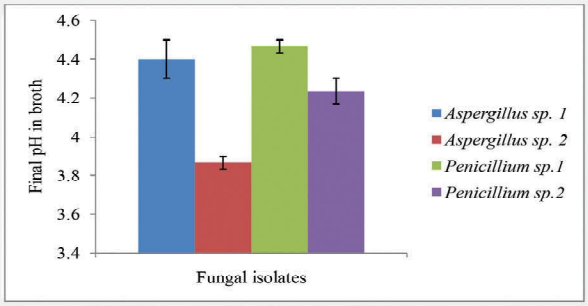

Fig.6: Differences in broth pH after inoculation of fungal isolates. Error bars represent standard error of standard deviation.

	Final pH of broths	Quantity of phosphates solubilised in broths (mgP_2O_5/ml)
Final pH of broths		
Pearson Correlation	1	-0.972**
Sig. (2-tailed)		0.006
N	5	5
Quantity of phosphates solubilised in broths (mgP2O5/ml)		
Pearson Correlation	-0.972**	1
Sig. (2-tailed)	0.006	
N	5	5

Table.1: Pearson's correlation between phosphate solubilized and final pH in broths.

Conclusion

The present study reports presence of potential phosphate solubilizer fungi in tea soil rhizosphere. Four fungal isolates belonging to genera *Aspergillus* and *Penicillium* were found to be efficient solubilizers of insoluble phosphates *in vitro*. These fungal isolates can therefore, serve as bio-inoculums for enhancing available phosphorus in agricultural lands. Despite decrease in pH along with phosphate solubilization, it was not only the sole factor for solubilization activity as the decrease in pH could not be correlated with solubilizing efficiency. Therefore, further works focusing on factors such as temperature of the environment, incubation periods and acids production by these isolates need to be established. Also, since the experiment was performed under laboratory conditions, pot experiments are necessary to confirm capabilities of these isolates as potential phosphate solubilizers.

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