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Evaluation of Phosphate Solubilizers from Soils of North Bengal and Their Diversity Analysis

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Abstract: Four hundred isolates obtained from soil samples collected from forest, river basin, agricultural fields and rhizosphere of plantation crops of North Bengal were screened for phosphate solubilizing activity on Pikovskaya's agar medium. Among the screened isolates, ninety showed phosphate solubilizing activity. Out of these, ten isolates belonging to *Aspergillus niger, A. melleus* and *A. clavatus* were selected for further studies. *In vitro* evaluation of phosphate solubilization by the different isolates using tricalcium phosphate (TCP) and rock phosphate (RP) revealed that the isolates could solubilize TCP better than RP. Selected isolates were mass multiplied using farm-yard manure (FYM) and were tested *in* vivo for their growth promoting activity in soybean. While all the isolates promoted growth, *A. niger* RSP-14 was found to be most effective. While the soil P content decreased due to the activity of the PSFs, root phosphate content showed an increase. These isolates were further analyzed for genetic variability. Genomic DNA from the fungal isolates were obtained and purified. UPGMA cluster analysis divided the ten isolates into two groups with the genetic similarity ranging from 0.35- 0.61. One group consisted of four isolates of *A. niger* and five isolates of *A. melleus,* while the other group had one isolate of *A. clavatus*.

Key words: Phosphate solubilizing fungi · *Aspergillus niger* · *A. melleus* · *A. clavatus*

INTRODUCTION

Fungi are important components of soil microbiota, typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions [1]. A wide range of soil fungi are reported to solubilize insoluble phosphorous. Strains of *Aspergillus niger* and *Penicillium* are the most common fungi capable of phosphate solubilization. Many bacterial, fungal, yeast and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied [2-5]. However, studies on genetic diversity of these important group of phosphate solubilizing fungi (PSF) are limited. Williams, *et al*. [6] and Welsh and McClelland [7] demonstrated the utility of single short oligonucleotide primers of arbitrary sequence for the amplification of DNA segments distributed randomly throughout the genome. Also Welsh and McClelland showed that the pattern of amplified bonds could be used for genome fingerprinting and Williams, *et al.* [6] showed that the differences (polymorphisms) in the pattern of bands amplified from genetically distinct individuals behaved as mendelian genetic markers named Random Amplified Polymorphic DNA (RAPDs). Most of the published studies on genetic characterization, detection of genetic variations and gene mutations were concentrated on the variations in chromosomes, isozyme polymorphism and biochemical diversity. A single set of arbitrary-sequence 10 mers may be used for fingerprinting any species. The many advantages of RAPD markers over RFLDs or isozyme markers accelerated the adoption of RAPD technology for the construction of genetic maps, fingerprinting and population genetic studies. Current reviews of the applications of RAPD technology are available. The utility of DNA markers as RAPD-DNA in detecting genetic variability among many phytopathogenic fungi have been recorded by various authors [8-10].

The present study was undertaken to test selected isolates of PSFs for their *in vitro* and *in vivo* activities and screen the random primers as effective molecular markers for genetic variability analysis among the isolates.

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MATERIALS AND METHODS

Isolation of Microorganisms: Soil samples were collected from three districts (Darjeeling, Jalpaiguri and Cooch Behar) of North Bengal. Source of soil samples includes forests (Sukhna, Lohagarh, Cinchona, Mongpong, Gorumara), river basin (Balasan, Mahananda, Dhorola, Torsa, Raidak) agricultural field (paddy, bamboo); rhizosphere of tea, rubber, mandarin (plantation crops) and *Cryptomeria.* Fungi from these soil samples were isolated using different techniques like Warcup's soil plate method [11]. Different types of media were used which included Potato Dextrose Agar (PDA), Potato Dextrose Agar-Rose Bengal (PDA- rose Bengal), Peptone dextrose agar, Elliott's Agar, Acid media, Richard's agar, Czapek's Dox agar, Asthana Howker's , Pikovskaya's agar (for screening phosphate solublizing activity).

Screening for Phosphorus Solubilizing Activity: Screening for primary phosphate solublizing activity of the isolates was carried out by allowing the fungi to grow in selective media, i.e., Pikovskaya's agar [12] for 7 to 10 days at 25°C. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solublizing activity of the fungus.

Evaluation of Phosphate Solubilizing Activity of Selected Isolates: Fungal isolates were grown in two sets of Pikovskaya's liquid medium (yeast extract, 0.50 g/L, dextrose, ammonium phosphate, 0.50 g/L, potassium chloride, 0.20 g/L, magnesium sulphate, 0.10 g/L, manganese sulphate, 0.0001g/L, ferrous sulphate, 0.0001 g/L, pH, 6.5) amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days. Fifty ml of the liquid medium was inoculated with 5 % v/v of the spore suspension prepared from 7 day old cultures grown on PDA slants and incubated at room temperature for 4-10 days with routine shaking at 100 rpm at 28°C in a rotary incubator. The initial pH of the medium was recorded. The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 rpm for 5 min. Quantitative estimation of phosphate was done following ammonium molybdate ascorbic acid method as described [13]. Amount of phosphate utilized or solubilized by the isolates were expressed as mg/ L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovskaya's liquid medium.

Mass Multiplication of PSF Isolates and Their Trial for Improvement of Plant Health: PSF isolates were grown separately in the PDA medium for sporulation over a period of 4-5 days after which harvested spore mass was suspended in sterile distilled water. For mass multiplication of the PSF, well decomposed FYM heaps were used. Spore suspension (100 ml) containing 106 spores / ml was used to inoculate 5 kg of FYM heaps. The FYM was first moistened slightly to optimize the PSF growth and kept in polythene bags in shade for 10 days. The mixture was regularly raked every third day during the total of this 10 days period. *Glycine max* was selected for trial with ten selected Phosphate solubilizers to assess their effect on growth in glass house conditions. Surface sterilized soybean seeds were sown in pots filled with the amended PSF. After germination and a few days of growth the physical parameters of the plants were monitored and the observations were noted after specific intervals.

Isolation of Genomic DNA: Fungi were grown in liquid media for 4 days and mycelia were harvested and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The aqueous phase was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min, the aqueous phase was then extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and then the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70 % ethanol by centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Purification of DNA: Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60 µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol as described earlier.

PCR Amplification: Four random decamer primers [OPB-2, OPB-3, OPB-6 and OPD-5] were used for RAPD analysis. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total

volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM random primer and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 35°C for 60 s and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 Advanced Gradient Thermocycler.

Gel Electrophoresis: PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water and then loaded in 0.8% agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

Analysis of the Obtained Data: RAPD profiles were scored by visually comparing RAPD amplification profiles and scoring the presence or absence of each band in each profile. Basically, the formation obtained from agarose gel electrophoresis was digitalized to a two - discrete - character - matrix (0 and 1 for absence and presence of RAPD - markers). UPGMA cluster analysis was carried following similarity coefficient matrix of reproducible bands using PC software NTSYS.

RESULTS AND DISCUSSION

Out of isolated fungi from the soil a total of 90 fungal isolates showed phosphate solubilizing activities as detected in Pikovskaya's agar medium by the appearance of halos around the inoculum on the medium. Ten isolates which showed maximum phosphate solubilizing activities in PVK agar medium were further tested for their activities in liquid medium using two types of inorganic phosphates, tricalcium and rock phosphate. *A. niger* (isolate RS/P-14) showed maximum solubilization of phosphorous (856 mg/l) whereas *A. clavatus* (isolate RHS/P-38) showed minimum of (799 mg/l) of phosphorous solubilization when the media were supplemented with tricalcium phosphate. When the medium was supplemented with rock phosphate, *A. melleus* (isolate RHS/R-12) showed maximum of 385 mg /L phosphorous solubilization and *A. clavatus* (isolate RHS/P-38) showed minimum of 288 mg/L phosphorous solubilization (Table 1) All the tested isolates increased growth in relation to control of which three isolates of *A. niger* were most effective (Figure 1). Phosphate level in the roots was found to be more in those plants treated with the amendments using isolates of *A. niger* (Table 2).

Table 1: Evaluation of phosphorus solubilization by fungal isolates in Pikovskaya's liquid medium amended with Tricalcium phosphate and Rock Phosphate

Organisms	Isolate nos.	P-solubilized (mg/L)	TCP*	RP **
Aspergillus niger	FS/L-04	856	366	
Aspergillus niger	RS/P-14	852	360	
Aspergillus niger	FS/L-40	847	370	
Aspergillus niger	FS/S-113	848	360	
Aspergillus melleus	RS/P-05	854	370	
Aspergillus melleus	RHS/R-12	810	385	
Aspergillus melleus	FS/L-13	817	381	
Aspergillus melleus	FS/L-17	820	379	
Aspergillus melleus	FS/L-18	821	376	
Aspergillus clavatus	RHS/P-38	799	288	

*Initial amount of tricalcium phosphate 997 µg/ml

**Initial amount of rock phosphate 500 µg/ml

Table 2: Phosphate content in root and soil of soybean plants after 20 days of inoculation

	P content (µg/g)	
Soil amended with	Root	Soil
A. niger (FS/L-04)	5.5	1.22
A. niger (RS/P-14)	5.8	0.99
A. niger (FS/L-40)	5.4	1.62
A. niger (FS/S-113)	3.7	1.44
A. melleus (RS/P-05)	4.4	1.54
A. melleus (RHS/R-12)	4.9	1.3
A. melleus (FS/L-13)	2.8	1.4
A. melleus (FS/L-17)	2.9	1.51
A. melleus (FS/L-18)	5.7	1.15
A.clavatus (RHS/P-38)	2.6	1.72
Unamended soil	0.7	1.79

In a similar study it was reported that isolates of *Aspergillus* and *Penicillium* isolated from agricultural soil showed maximum level of phosphate solubilization activity *in vitro* when liquid medium was supplemented with both tricalcium phosphate and rock phosphate separately [14]. DNA samples preparation before RAPD-PCR amplification was found crucial for fingerprint. The yield of DNA was determined spectrophotometrically as 24 µg/g of mycelial mat. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio was 1.8 and DNA quantity was evaluated by 0.8% agarose gel electrophoresis. The PCR conditions for RAPD analysis were optimized by investigating each factor individually. This included genomic DNA quality and concentration, primer annealing and extension temperature as well as

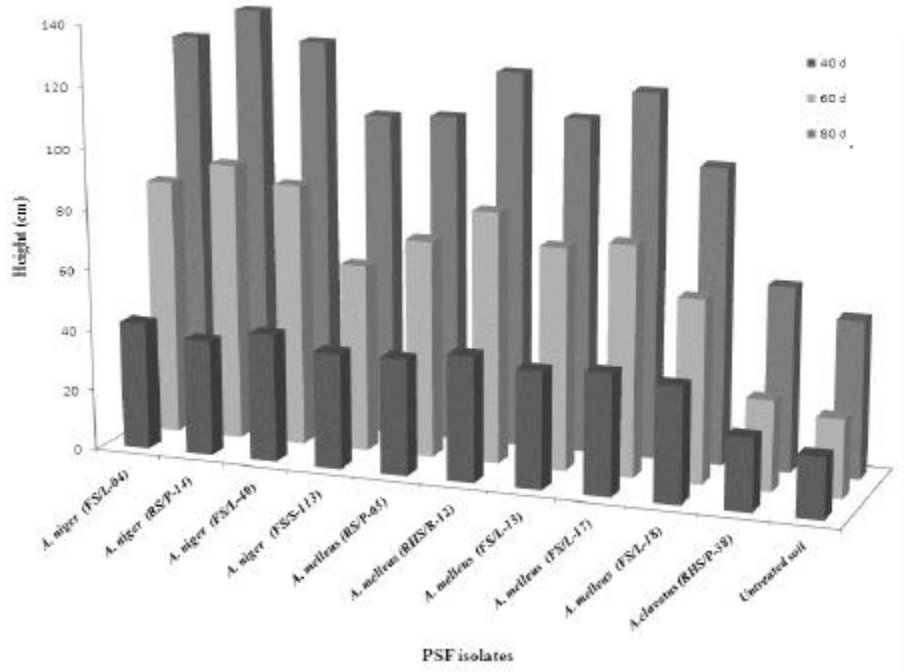

Fig. 1: Effect of amendment of soil with phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* on growth of soybean plants

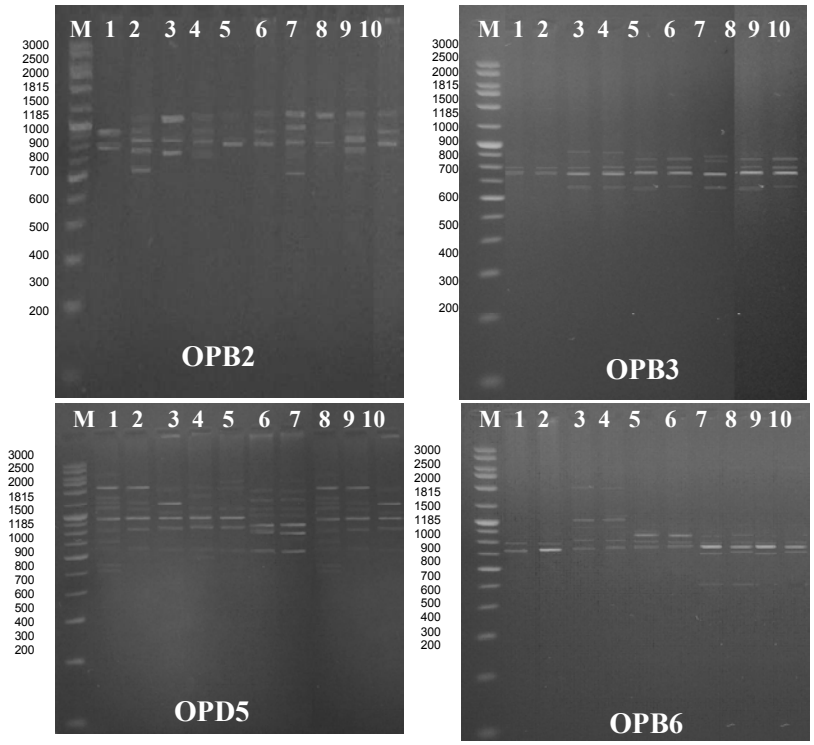

Fig. 2: RAPD amplified products of phosphate solubilizing isolates of *A. niger, A. melleus* and *A. clavatus* using four random primers

Lane M: Low range DNA marker, Lane 1: *A. niger* (FS/L-04), Lane 2: *A. niger* (RS/P-14), Lane 3: *A. niger* (FS/L-40), Lane 4: *A. niger* (FS/S-113) , Lane 5: *A. melleus* (RS/P-05), Lane 6: *A. melleus* (RHS/R-12), Lane 7: *A. melleus* (FS/L-13), Lane 8: *A. melleus* (FS/L-17), Lane 9: *A. melleus* (FS/L-18), Lane 10: *A. clavatus* (RHS/P-38)

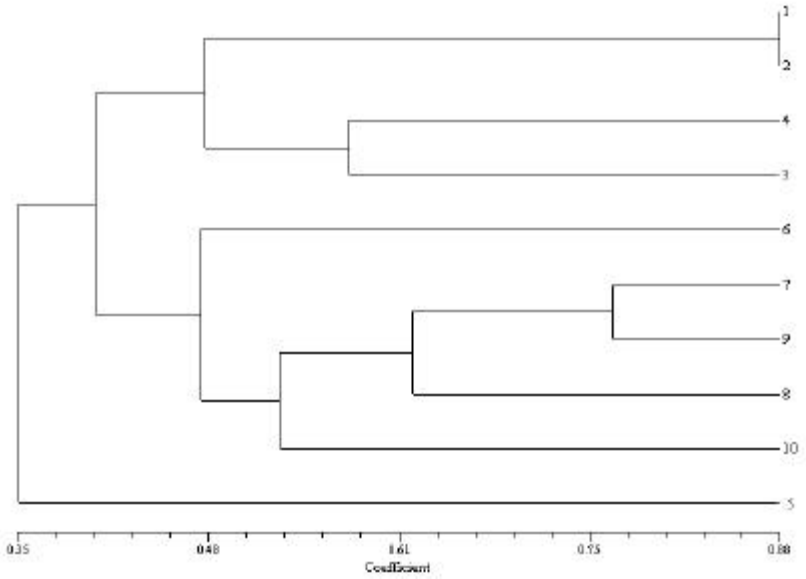

Fig. 3: Dendrogram showing the genetic relationships among 10 Phosphate solubilizing fungal isolates based on RAPD analysis [1: *A. niger* (FS/L-04), 2: *A. niger* (RS/P-14), 3: *A. niger* (FS/L-40), 4: *A. niger* (FS/S-113) , 5: *A. melleus* (RS/P-05), 6: *A. melleus* (RHS/R-12), 7: *A. melleus* (FS/L-13), 8: *A. melleus* (FS/L-17), 9: *A. melleus* (FS/L-18), 10: *A.clavatus* (RHS/P-38)

denaturation time and temperature. It was found that quality of genomic DNA extracted as described here was a good template for PCR amplification. In the present investigation, four random decamer primers - OPD-5, OPB-2, OPB-3 and OPB-6 gave sufficient polymorphism among the isolates of *A. clavatus*, *A. niger* and *A. melleus*. The amplified fragments ranged from 1100 to 600 bp in size. A total of 127 polymorphic bands were obtained with an average of 31.75 bands/ primer (Figure 2). The second group consisting of two isolates of *A. clavatus*, two isolates of *A. melleus* and two isolates of *A. niger* showed another sub group at 50 percent similarity. The selected isolates showed three different lineages at sixty one percent similarity level (61%). During the past few years, numerous publications demonstrated the utility of RAPD markers for the analysis of the genetic diversity among species and within fungi populations and plant populations [15-18]. In studies conducted with the toxin producing *Aspergillus* strains, it was found that *A. flavus* and *A. parasiticus* did not show any relationship between RAPD-based band profile and toxin production [19]. Results of the present study suggest that these random RAPD markers can be used for identification of phosphate solubilizers. The variation obtained in terms of their genetic make-up gives an idea of improving the marker based selection of this beneficial group of organisms.

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