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Characterization of *Fusarium Equiseti* KUSF0105 with Respect to in-vitro Phosphate Solubilization under Varying Cultural Parameters

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ABSTRACT

Presence of soluble phosphorous in the soil is an important determining factor for the overall growth and the development of the agricultural crops. Phosphate solubilizing microorganisms are the potential candidates for releasing the soluble phosphates from its bound complexes and thus make them available to the plants in utilizable forms. The present study was conducted to determine the effects of various culture parameters on the phosphate solubilization ability of the nonpathogenic *Fusarium equiseti* MF803160 isolated from soil sample of an agricultural field of Bamnabad village in Raninagar block II, Murshidabad, West Bengal, India. Nonpathogenic nature of the isolate was established by performing pathogenecity test on several seed plants. High phosphate solubilisation index (1.52) was found on Pikovskya agar medium. Phosphate solubilization was estimated by spectrophotometric method using molybdate vanadate reagent. Maltose (1800 ppm) and ammonium sulphate (1800 ppm) respectively were found to be most stimulatory in phosphate solubilization by the *Fusarium* isolate. Ammonium chloride also influenced phosphate solubilization nearly to the same extent (1780 ppm). Significantly, in acidic pH 4 (1830 ppm) and at 27°C temperature (1620 ppm), phosphate solubilization was found to be most satisfactory. Notably, with the increased in pH, phosphate solubilization declined gradually. Growth and phosphate solubilization of the isolate were completely checked at 37ºC. Thus, the *Fusarium* isolate could be exploited in agricultural fields as a potential phosphate biofertilizer.

Keywords: Phosphorous, Fusarium, pathogenecity test, solubilisation index, molybdate vanadate reagent, maltose, ammonium chloride, pH, temperature, phosphate biofertilizer etc.

1. INTRODUCTION

Phosphorus (P) is one of the most essential macronutrients required for growth and development of plant. It exists in two forms in soil, as organic and inorganic phosphates. However, approximately 95–99% of soil phosphorus is present in the insoluble form and hence cannot be utilized by the plants (Vassileva et al., 1998). The problem of P deficiency is generally alleviated through the application of P fertilizers. But after application, a large proportion of fertilizer phosphorus is quickly bound to metal cations to from insoluble inorganic salts (Omar, 1998). Therefore, very little fraction of the applied phosphorus is utilized and continuous application becomes obligatory (Abd Alla, 1994). Moreover, repeated and injudicious applications of chemical P fertilizers lead to the loss of soil fertility by disturbing microbial diversity, and consequently reduces yield of crops. To circumvent phosphorus

deficiency, phosphate-solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner.

Fungi are important components of soil microbiota typically constituting more of the soil biomass. A wide range of soil fungi are reported to solubilize insoluble phosphorous. In soil, P-solubilizing fungi constitute about 0.1 – 0.5% of total fungal population. Many soil fungi are reported for actively converting insoluble phosphates to soluble primary and secondary orthophosphate ions solubilization and mineralization (Bishop et al. 1994). These phosphate solubilizing microorganisms (PSMs) assimilate phosphate, solubilize them and thus make it available to plants which in turn are assimilated for the increase in the yield of crop (Toro et al., 1997). Several theories have been proposed to explain the mechanisms of microbial solubilization of P. Broadly these theories have been categorized into three groups: (i) the organic acid theory, (ii) the sink theory, and (iii) the acidification by H^+ excretion theory (Khan et al., 2010). In the well recognized and accepted organic acid theory, the insoluble sources of P are solubilized by P-solubilizing organisms either by: (a) lowering the pH, or (b) by enhancing chelation of the cations bound to P. A variety of organic acids such as lactic acid, maleic acid, malic acid, acetic acid, tartaric acid, citric acid, fumaric acid and gluconic acid have been reported to produce by a group of soil fungi including *F. oxysporum* to solubilize inorganic phosphate (Akintokun et al., 2007). Other organic acids involved in the P solubilization are α-ketogluconic acid, glycolic acid, oxalic acid, succinic acid, and propionic acid. In the sink theory, P-solubilizing organisms remove and assimilate P from the aqueous medium and hence, activate the indirect dissolution of calcium phosphate compounds by consistent removal of P from broth culture medium. Mineralizations of most organic phosphorous compounds were carried out by production of enzymes: (i) non-specific phosphatases, which dephosphorylate phospho-ester or phosphoanhydride bonds of organic matter, (ii) phytases, which specifically cause release of P from phytic acid, and (iii) phosphonatases and C-P lyases that cleave the C-P of organophosphonates (Rodriguez et al. 2006). Many rhizospheric microorganisms are involved in the mineralization of organic phosphorus through the action of phosphatases (Tarafdar et al., 1988). Developing fungal inoculants with high phosphatase and phytase activity would be of great practical interest for augmenting plant nutrition and reducing P pollution in soil. The present study aims at evaluating the *Fusarium* isolate in terms of its phosphate solubilization under various laboratory parameters with a view to foresee the potential of the isolate to be exploited in agricultural sectors.

2. MATERIALS AND METHODS

2.1. Collection of soil sample: A soil sample was collected from the rhizospheric region of the rice plant located in Lochanpur village of Raninagar block 2 in Murshidabad district, West Bengal, India. The agricultural field was placed in close vicinity of Padma river near Indo-Bangladesh boarder region and cultivated for several crops throughout the year where no *Fusarium* diseases were reported previously.

2.2 Isolation and identification of fungus: The soil was screened for isolation of the fungi by dilution plate technique on selective peptone PCNB agar medium [composition (g/l): peptone 15, KH_2PO_4 1.0, $MgSO_4$, $7H_2O$ 0.5, PCNB 1.0, agar 20, pH 6] supplemented with streptomycin sulphate 1.0 g/l and neomycin sulphate 0.12 g/l. The plates were incubated at 28°C for 5-7 days until visible sign of colony growth occurred. Fungal isolates were identified by observing their colony morphology, sporulation and pigmentation on Czapek's Dox agar (CDA) medium. One promising isolate was further identified based

on rDNA gene analysis. For this, genomic DNA was extracted and used as template for amplification of the rDNA region using the primer pair LROR (TCCGTAGGTGAACCTGCGG) and LR5 (GCTGCGTTCTTCATCGATGC). The amplified product was sequenced and the sequence was analysed using Nucleotide BLAST function at NCBI to find similarity of the sequence with nucleotide database. Phylogenetic tree was constructed using the software MEGA6. Multiple sequences alignment was carried using CLUSTALW and the evolutionary history was inferred using the Neighbour-joining method.

2.3. Pathogenecity test: Effect of culture filtrate on germination of seeds of six plants such as chickpea (*Cicer arietinum*), black gram (*Vigna mungo*), cucumber (*Cucumis sativus*), chilli (*Capsicum* sp.), mustard (*Brassica* sp.) and paddy (*Oryza sativa*) was evaluated to check whether the isolate was pathogenic or not. The isolate was cultivated in CD broth for 14 days and their culture filtrate was used for the study. Ten seeds were taken for each experimental set up. Seeds were surfaced sterilized in 0.1% $HgCl_2$ solution for 1 min and washed thrice with sterile water. The surface sterilized seeds were then transferred to culture filtrate of the *Fusarium* isolates and kept at 4°C for overnight period. On the subsequent day, the seeds were placed on sterilized pre-soaked blotting paper kept within the petridish. After five days, percentage of seed germination, length of radical and plumule were recorded. Vigour index was also calculated using following formula:

Vigour index (VI) = root length+ shoot length× germination%

2.4. Assay of phosphate solubilization: The *Fusarium* isolate were inoculated on Pikovskaya's agar medium and incubated at 28°C for 7 days. The fungal isolate showed the clear zones around the colony and was thus considered as positive (Pikovskaya, 1948). The solubilization index [the ratio of the total diameter (colony + halo zone) to the colony diameter] was measured (Premono et al., 1996).

The *Fusarium* isolate was used for quantitative estimation of the soluble phosphates produced in the Pikovskaya's (PVK) broth medium. After incubated at 28°C for a week, 1 ml each of the culture filtrate and the uninoculated broth (control) were mixed with 3 ml of distilled water and 1 ml of molybdate vanadate reagent. After 20 min of incubation at room temperature, the absorbance at 470 nm was measured using a spectrophotometer (Jeon et al., 2003). Amount of solubilised phosphate was determined using the standard curve of KH_2PO_4.

2.5. Effect of various carbohydrate and nitrogen sources on phosphate solubilization: Different sets of modified (without carbohydrate) Pikovskaya's broths were prepared amended with 1% of the respective carbohydrate source viz., dextrose, sucrose, maltose, lactose, sorbitol. Likewise different sets of modified (without nitrogen) Pikovskaya's broths were prepared amended with respective nitrogen source viz., sodium nitrate, sodium nitrite, ammonium nitrate, ammonium chloride, ammonium sulphate. Control sets without carbohydrate or nitrogen were also prepared. The *Fusarium* isolate was inoculated and incubated at 28°C for 14 days. Amount of soluble phosphate in the broths were estimated by spectrophotometric method using molybdate vanadate reagent. Data of the mycelial dry weights of the different treatments were also kept.

2.6. Study of effect of different pH and temperature on phosphate solubilisation: Different sets of Pikovskaya's broths were prepared adjusting pH range from 4-10 with dilute HCl and NaOH. Five mm mycelial disc of the promising *Fusarium* isolate was inoculated in the broth and kept at 28°C for 14 days. Four sets of Pikovskaya's broths were also inoculated by the same *Fusarium* isolate and incubated

at four different temperatures viz., 14°C, 27°C, 32°C and 37°C for 14 days. Amount of soluble phosphate in the broths were estimated by spectrophotometric method using molybdate vanadate reagent. Mycelial dry weights of the different treatments were also taken.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of the fungus: On selective peptone PCNB agar medium the several fungal colonies with similar morphology had appeared. One fungal isolate, designated as KUSF0105 was selected for further study. On CDA medium the fungal isolate showed white, circular, compact, smooth, fast growing colony and developed light yellow pigment (Fig. 1). It abundantly produced falcate, curved medium sized (40-50 µm X 3.75-4.25 µm) macroconidia having 3-6 septa with curved or pointed apical cell and foot shaped basal cell with a notched or a rounded end (Fig. 1). The dorsal side was found to be more curved than the ventral side. Microconidia were sparse, ellipsoid to fusiform; short sized (8.25-12 µm X 1.0-2.5 µm) having 1-2 septa. Chlamydospores were lacking. Molecular identification of the fungal isolate KUSF0105 was performed based on rDNA sequence analysis. ~ 544 bp amplicon of rDNA region of the isolate was observed on agarose gel and a stretch of 315 bp had been sequenced. Search for sequence homology through nucleotide BLAST function in NCBI database was performed and maximum identity (99%) was found with the rDNA sequence of *Fusarium equiseti*. When a phylogenetic tree was constructed by Neighbour-joining method based on rDNA sequence of KUSF0105 and other similar sequences obtained from BLAST search and one outgroup as *Alternaria* sp., relatedness of KUSF0105 with related fungal species was observed and it belonged to the same evolutionary branch with *Fusarium equiseti* (Fig. 2). Based on all these key specifics, the fungal isolate was identified as *Fusarium equiseti*. The partial rDNA region of the fungal isolate KUSF0105 had been submitted to the genebank under the accession no. MF803160.

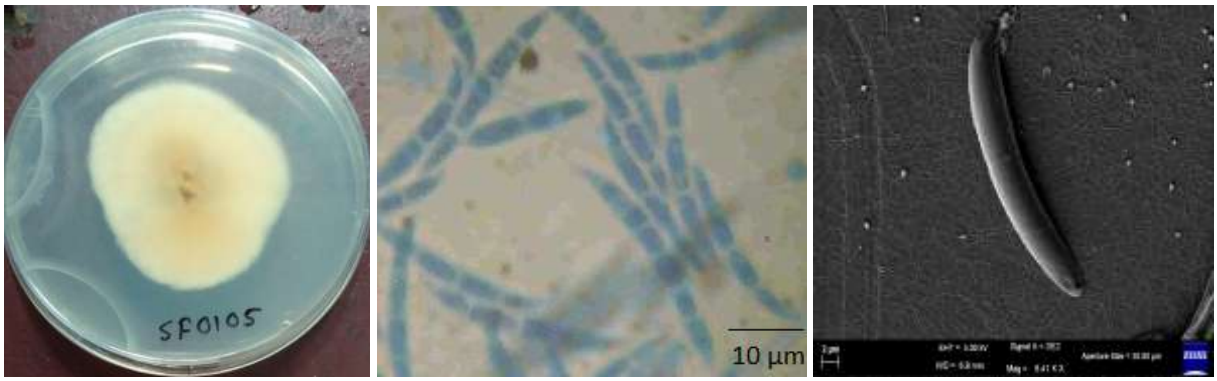


Fig. 1: Reverse side of growth of *Fusarium equiseti* MF803160 on CDA medium after 7 days (left); Microscopic field of *Fusarium equiseti* MF803160 showing macroconidia stained with cotton blue (middle); SEM photograph of a single macroconidium of *Fusarium equiseti* MF803160 (right)

3.2. Pathogenecity test: Effect of culture filtrate on seed germination and vigour index of the tested seed plants have been depicted in table 1. Most of the seeds showed germination although percentage of germination varied among plants. The culture filtrate of the particular *Fusarium equiseti* MF803160 did not show any inhibitory effects on germination of the tested seed plants thus establishing the nonpathogenic nature of the isolate. Moreover growth stimulatory effects of the fungal culture filtrate on the three plants viz., black gram, chili and paddy were evidenced by the increase in percentage of seed

germination and vigour index. Seed germination in all the inoculated treatments was conspicuously comparable to the control. There was also early onset of seed germination in few inoculated seeds as well.

Table 1. Pathogenecity Test of *Fusarium equiseti* MF803160

Isolate no.	Chickpea		Blackgram		Cucumber		Chili		Mustard		Paddy	
	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index	% of germination	Vigour index
Fusarium equiseti MF803160	70	15.4	100	31.9	65	94.2	50	32.2	70	61.5	90	22.6
Control	80	23.2	100	28.4	70	119.7	60	40.2	70	38.5	80	16.2

3.3. Assay of phosphate solubilization: The Fungal isolate showed phosphate solubilizing activity as detected in Pikovskaya's agar medium by the appearance of halos around the colony on the medium (Fig. 2). High solubilization index of 1.52 was recorded was found in *Fusarium equiseti* MF803160. Thus, phosphate solubilization characteristic of the isolate was studied further.

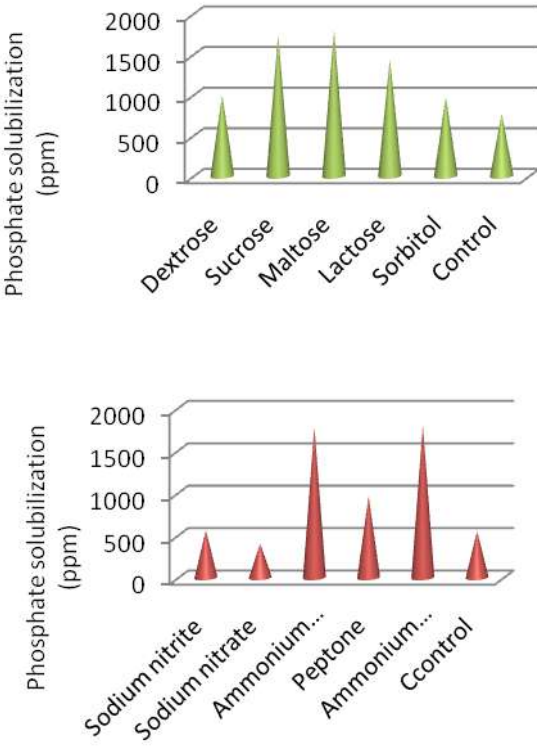
Fig.2. Formation of halo around the colony on Pikovskaya's agar medium due to solubilization of tricalcium phosphate by *Fusarium equiseti* MF803160

3.4. Effect of various carbohydrate and nitrogen sources on phosphate solubilization: *Fusarium equiseti* MF803160 was tested for its phosphate solubilisation property in modified Pikovskaya's broth containing any of the five carbohydrates or nitrogen sources (Table 2; Fig. 4). Maximum growth and phosphate solubilization were observed in PVK broth containing maltose or $(NH_4)_2SO_4$. Thus expression of this property was also dependent on growth of the organism.

Table 2. Phosphate solubilisation of *Fusarium equiseti* MF803160 in presence of various carbohydrate and nitrogen sources

Sl no	Carbohydrate source	Mycelial dry weight (g)	Phosphate solubilization (ppm)
1.	Dextrose	0.145	1000
2.	Sucrose	0.157	1740
3.	Maltose	0.292	1800
4.	Lactose	0.190	1450
5.	Sorbitol	0.166	980
	Control	0.090	770
	Nitrogen source		
1.	$NaNO_2$	0.121	560
2.	$NaNO_3$	0.162	405
3.	NH_4Cl	0.150	1780
4.	Peptone	0.205	960
5.	$(NH_4)_2SO_4$	0.219	1800
6.	Control	0.065	550

Fig. 4. Effect of various carbohydrate (upper) and nitrogen (lower) sources on phosphate solubilization of *Fusarium equiseti* MF803160

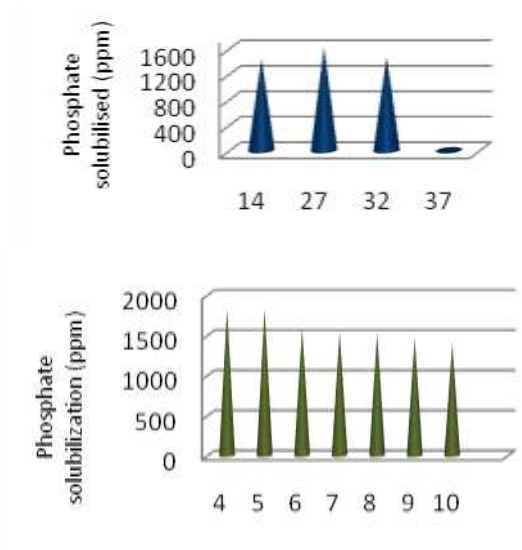


3.5. Effect of different temperature and pH on phosphate solubilization: *Fusarium equiseti* MF803160 was tested for its phosphate solubilization property in Pikovskaya's broths incubated at different temperatures and in varying pH range (Table 3; Fig. 5). Maximum growth and phosphate solubilization were observed in culture incubated at 27°C. Phosphate solubilization increased at acidic pH, although growth was maximum at pH 8, the phosphate solubilization was comparatively lesser that that at pH 4.

Table 2. Phosphate solubilization by *Fusarium equiseti* MF803160 at varying temperature and pH

Growth conditions		Mycelial dry wt. (g)	Phosphate solubiliza-tion (ppm)
Temperature (°C)	14	0.090	1440
	27	0.166	1620
	32	0.099	1470
	37	-	-
pH	4	0.115	1830
	5	0.126	1820
	6	0.116	1580
	7	0.178	1540
	8	0.195	1520
	9	0.136	1460
	10	0.147	1400

Fig. 5. Effect of temperature (upper) and pH (lower) on phosphate solubilization of *Fusarium equiseti* MF803160



4. DISCUSSION

Phosphate solubilising microorganisms may contribute to plant nutrition by liberating P from insoluble phosphates compounds. Mineralization of most organic phosphorous compounds were carried out by production of enzymes, such as phosphatase, phytase, phosphonoacetate hydrolase, D-α-glycerophosphatase, C-P lyase and solubilization of inorganic phosphates, such as tricalcium phosphate was found to be involved with acidification of the medium via biosynthesis and release of a wide variety of organic acids, such as gluconic acid, 2-ketogluconic acid, acetic acids, glycolic acid, oxalic acid, malonic acid, succinic acid, citric acid and propionic acid (Akintokun et al., 2007). The phosphate solubilization ability of microorganism is related to its organic acid production; however nature of acid produced is also important (Vassileva, 1998). The *Fusarium* isolate was found to be potential phosphate solubilizer and produced clear halo around their colony on Pikovskaya's agar medium (Fig. 2). The ability of the microorganisms to solubilize insoluble phosphates is usually expressed phenotypically (Perez et al. 2007). Phosphate solubilization index of endophytic *Fusarium* isolates was recorded as 1.28-1.81 (Sahoo and Gupta, 2014). *Fusarium equiseti* MF803160 showed high solubilization index (1.52) and was used for estimation of solubilized phosphate from inorganic phosphate under various cultural parameters using molybdate vanadate reagent. Temperature, pH, carbohydrate and nitrogen sources were the different parameters based on which phosphate solubilization abilities of PGPF were evaluated. Nitrogen and carhydrate in the culture medium played significant roles in solubilisation of inorganic phosphate (Nahas, 1996). Among the carbohydrate and nitrogen sources used, maltose (1800

ppm) and ammonium sulphate (1800 ppm) respectively were found to be most stimulatory for solubilization of inorganic phosphate by *Fusarium equiseti* MF803160 (Table 1). Glucose and maltose decreased the pH of the medium to maximum extent and caused highest solubilization of phosphorus, followed by sucrose, xylose and galactose in *Aspergillus* sp and *Penicillium* sp. (Pradhan and Shukla, 2005). Ammonium chloride also influenced phosphate solubilisation nearly to the same extent (1780 ppm). Similar results were obtained by Mujahid et al. (2015). A number of fungi and bacteria have been reported of being able to solubilized phosphate only in the presence of ammonium as the nitrogen sources (Illmer and schinner, 1992). Significantly, in acidic pH and temperature 27°C, phosphate solubilisation was found to be most satisfactory (Table 2). Significantly, with the increased in pH, phosphate solubilization was found to be gradually declining. Phosphate solubilization was completely checked at 37ºC might be due to cessation of growth. Kang (2002) reported optimum temperature for insoluble inorganic phosphates solubilization by a soil-inhabiting fungus *Fomitopsis* sp. PS 102 was 28°C. *Fusarium* species were also reported by Elias et al. (2016) and Yasser et al. (2014). Radhakrishnan (2015) found increased phosphate solubilization in *F. verticillioides* RK01 by increasing activities of acid phosphatase, alkaline phosphatase and fungal biomass.

5. CONCLUSION

Phosphate solubilizing fungi are an important contributor in microbial P-mobilization and would be important possible way to increase available P for plants. The present study revealed higher phosphate solubilization efficiency of *Fusarium equiseti* MF803160 which could be used as potential phosphate biofertilizer for promoting growth of different crop. Thus we can conclude that the amendment of soil with TCP along with the application of P-solubilizing nonpathogenic *Fusarium equiseti* MF803160 is a sustainable way for increasing crop yield and also improving the physio-chemical properties of the soil. However, in vivo studies can only justify the potential of any phosphate solubilizing strain in promotion of plant growth and their establishment.

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