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Screening of Phosphate Solubilizing Fungi (PSF) Isolated from Cash-Crop Rhizosphere Soils

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ABSTRACT

Phosphate solubilizing fungi (PSF) were isolated from the rhizosphere soils of cash-crop plants such as sunflower, cotton, chilly, tomato, black gram, sorghum, brinjal, green gram, okra, and red gram. Based on the solubilization zone formation in the Pikovskaya's agar medium, PSF was isolated. The isolated fungi were identified up to species level. The PSF were screened for their P-solubilization capacity under *in vitro* conditions. The isolates differed in solubilization zone formation, pH reduction, production of organic acid, phosphatase enzyme and available phosphorus. The ability of solubilization zone formation, pH reduction, production of organic acid, phosphatase reflected in the solubilization capacity of PSF isolates. The ability of phosphate solubilization by PSF differed between the isolates with tricalcium phosphate and rock phosphate as the phosphate source. PSF isolates possessed greate r ability to solubilize the tricalcium phosphate (TCP) than rock phosphate (RP).

INTRODUCTION

Phosphorus is one of the major plant nutrients required in optimum amount for proper plant growth and also known to involve many functions in the plant growth and metabolism. Several important cellulars, metabolic and reproductive functions rely on sufficient phosphorus supply. Only about 25 percent of the phosphorus applied to the soil is available for the crops and the rest become unavailable due to chemical fixation with aluminum and iron in acidic soils. Indian soils are characterized by poor and medium status with respect to available phosphorus [1]. Phosphorus ranks next to nitrogen is important for living plants.

A greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and cannot be utilized by the plants [2]. To increase the availability of phosphorus for plants, large amounts of fertilizer are being applied to soil. But a large proportion of fertilizer phosphorus after the application is quickly transformed to the insoluble form [3]. Therefore, the very little percentage of the applied phosphorus is available to plants, making continuous application necessary [4]. However, phosphorus deficiencies are widespread on soil throughout the world and phosphorus fertilizers represent a major cost for agricultural production. Many soil fungi and bacteria are known to solubilize inorganic phosphates [5].

PHOSPHATE SOLUBILIZING MICROORGANISMS (PSMS)

PSMs play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Microorganisms are involving in a range of process that affects the transformation of soil phosphorus (P) and thus is an integral component of the soil ‘P' cycle. Many bacterial, fungal, yeast and actinomycetes species capable of solubilizing sparingly soluble phosphorus in pure culture have been isolated and studied [6]. Some heterotrophic bacteria and fungi are known to have the ability to solubilize inorganic phosphate from insoluble sources and made available to plants. These microorganisms are known as phosphate solubilizers. The most efficient phosphate solubilizing bacteria include *Bacillus* and *Pseudomonas* and that of fungi include species of *Aspergillus* and *Penicillium* [7].

Phosphate-solubilizing microorganisms are recognized as a solution to the challenges in P fertilization management due to their abilities to mobilize P from recalcitrant sources [8]. In general, phosphate-solubilizing fungi (PSF) possess greater abilities to release P from recalcitrant inorganic P, when compared to bacteria [9]. To date, most representative strains

of *Aspergillus* spp. and *Penicillium* spp. have been widely reported as P solubilizers [10] and the stability of P-solubilizing capability of these fungi has been noted to be superior to that of P-solubilizing bacteria upon repeated sub-culturing in the synthetic medium [11]. Moreover, PSF has been observed to secrete more acids than bacteria and display greater P-solubilizing activity. Several soil fungi, particularly those belonging to the genera *Penicillium* and *Aspergillus* possess the ability to bring insoluble soil phosphates into soluble forms by secreting weak organic acids such as formic, acetic, propionic, lactic, gluconic, fumaric and succinic acid.

MATERIAL AND METHODS

ISOLATION AND ENUMERATION OF PSF

Soil and root samples were collected from crop plants such as sunflower, cotton, chilly, tomato, black gram, sorghum, brinjal, green gram, okra, and red gram. The soil samples were air dried under shade and used for the isolation and enumeration of PSF. Isolation of P-solubilizing fungi was carried out by serial dilution and pour plate technique. Pikovskaya's agar amended with tricalcium phosphate was used to isolate the P-solubilizing fungi. The clear halo zone around the fungal colonies was considered as a positive result for P-solubilization. Distinct colonies present on the plates were selected, purified by repeated culturing and maintained on PDA slants at 4ºC.

IDENTIFICATION OF FUNGAL ISOLATES

The fungal isolates were grown on potato dextrose agar medium for one week at 28±2°C and the colony characters were recorded. The color, type, and shape of the fungal colonies were studied. The fungal cultures were identified on the basis of colony characteristics and microscopic examination.

CHARACTERIZATION OF PSF ISOLATES

Phosphate solubilization in the solid medium

The PSF strains were inoculated in solid hydroxyapatite medium as disc and incubated for 7 days. After the incubation period, the diameter of the halo region produced around the colonies was measured.

Change in pH of the medium

The selected PSF strains were grown in PDA medium and inoculated as disc into Pikovskaya's broth. After the incubation period, the pH was measured at different period of growth.

Estimation of organic acids

The organic acid produced by PSF strain was quantified in terms of total titrable acidity (TTA) of the culture filtrate. The total titrable acidity was expressed by ml of 0.01 N NaOH consumed [12].

Estimation of phosphatase activity

The PSF isolates were grown in Pikovaskaya's broth where TCP was replaced with the organic source (p-glycerophosphate). The phosphatase activity was expressed μ moles of PNP released/ml of filtrate/hour [13].

Estimation of available Phosphorus

The available phosphorus in the culture filtrate was estimated following the method of Olsen [14].

RESULTS

ISOLATION OF PSF

Based on the solubilization zone production in the solid medium, two fungal isolates from each crop plants were selected and totally 20 PSF were selected. The fungal isolates were maintained in PDA slants and used for further studies. The result revealed that the population level of PSF was higher in rhizosphere soils collected from tomato plants and least in the rhizosphere soils of sorghum plants (Table 1).

IDENTIFICATION OF FUNGAL STRAINS

Based on the morphological, biochemical and microscopical tests, the PSF were identified up to species level. Among 20 phosphates solubilizing fungal 9 isolates were identified as *Aspergillus flavor*, 7 isolates as *A. niger* and 4 isolates as *Penicillium notatum* (Table 2).

CHARACTERIZATION OF PSF

The isolated 20 PSF were characterized under *in vitro* conditions. The fungal isolates were subjected various *in vitro* tests. There were marked difference was observed between the PSF isolates. They differed in P solubilization zone formation, pH change, organic acid, and phosphatase enzyme production and capable of production of available phosphorus from the insoluble P sources such as tricalcium phosphate (TCP) and rock phosphate (RP) (Table 3 and 4).

Phosphate solubilization zone formation

The phosphate solubilization zone was estimated by measuring the solubilized zone produced by the PSF isolates. 'P' zone was higher with the isolate from the green gram (GGF2) in the presence of TCP and also higher with TF1 and RGF2 with RP as P source. There was not much variation in the zone formed between other PSF isolates.

Change in pH of the medium

All the PSF brought down the pH of the culture medium from 7.0 as in the control medium. Among 20 PSF isolates, maximum reduction was observed with GGF2 isolated from green gram in the presence of TCP as P source. In the case of rock phosphate, the reduction was higher with the isolate RGF2.

Organic acid production

PSF isolates GGF2 (29.7 0.1N NaOH consumed) produced more organic acid followed by TF1 (27.8 0.1N NaOH consumed) with TCP. With RP, organic production was higher in RGF2 (21.7 0.1N NaOH consumed) followed by TF1 (18.1 0.1N NaOH consumed).

Phosphatase activity

The phosphate sources such as TCP and RP greatly affected the phosphatase activity. The activity was higher with GGF2 and RGF2 in the presence of TCP and RP respectively.

Available Phosphorus

The production of organic acids and phosphatase enzyme had positive relationships with the available P content. The isolates those produced higher organic acid and phosphatase, also

able to solubilize the P source such as TCP and RP. The P solubilization potential of selected PSF isolates was tested *in vitro* by estimating available phosphorus in the culture medium. The results indicated there was a wide variation in the phosphate solubilization capacity of different fungal isolates. Among 20 PSF, GGF2 released more phosphorus (50 ppm) in the medium followed by RGF2 (42 ppm) with TCP and the isolate RGF2 was able to solubilize higher phosphate source (40 ppm) followed by TF1 (38 ppm) in the presence of RP.

DISCUSSION

POPULATION DYNAMICS AND IDENTIFICATION OF PSF

The phosphate solubilizer fungi isolates were identified based on their colony morphology *ie*., pigmentation, shape, size, texture, elevation, and margin. A total of 359 fungal isolates were obtained from 150 rhizosphere soil samples from different plants such as cabbage, faba bean, haricot bean, sugar cane, and tomato. Out of the isolated fungi, a total of 167 phosphate solubilizing fungal cultures having the potential of phosphate solubilization were isolated. Of the isolates, the highest numbers of PSF (28.14%) were recovered from tomato and the least (13.17%) was obtained from faba bean [15]. *Penicillium* and *Aspergillus* spp. are the dominant P-solubilizing filamentous fungi found in rhizosphere [16]. Filamentous fungi are highly important in RP solubilization. They are widely used as producers of an organic acid. *Aspergillus niger* and some *Penicillium* species have been tested for solubilization of RP and other biotechnological importance such as biocontrol, biodegradation and phosphate mobilization [17].

The richness of P-solubilizing fungi in the rhizosphere soil could be probably because of sufficient root exudates since phosphate solubilizing microorganisms are mainly dependent on carbon-rich sources from plant root for active production of organic acids that are utilized for solubilizing soil-bound phosphate. *Aspergillus* spp. were the most frequently occurring P-solubilizing fungi of the three identified genera. This may be due to the efficiency of *Aspergillus* sp. in root colonization [18]. The current result is in agreement with the earlier findings of several workers [19] who observed the predominant occurrence of P solubilizing fungi belonging to genus *Aspergillus* followed by *Penicillium* spp. in the rhizosphere of different crop plants.

MECHANISM OF P SOLUBILIZATION

The maximum solubilization index was shown by different PSF isolates. The variable potential of phosphate solubilization based on SI (Solubilization Index) on agar plate may be because of the varying type, amount and diffusion rates of diverse organic acids secreted by fungal isolates [20]. The solubilization indices of the test phosphate solubilizing fungal strains (*Penicillium italicum* and *Aspergillus niger*) were observed 2.42 and 3.15 respectively [21]. Conversely, SI for different fungal strains isolated from sugarcane and sugar beet which ranged from 1.13 to 1.59 [22] and also SI ranged from 1.53 to 1.80 for the fungal cultures isolated from maize rhizosphere [23]. The periodical estimates of P in broth media revealed the potential of the isolates in releasing P from insoluble phosphate sources. The fungal isolates solubilize the insoluble phosphate sources such as TCP and RP with a gradual increase in the middle of the incubation period. Maximum solubilization of phosphate occurring at day 15 of incubation for TCP under controlled conditions. In rock phosphate solubilization indicated that the maximum soluble phosphorus release was on the tenth day of incubation by the PSF isolates in the liquid culture medium, while maximum solubilization of RP from nine to twelve days after incubation.

The fungal isolates were able to release a considerable amount of P when the medium was supplemented with rock phosphate. This indicates the potential of these fungal isolates insolubilization of insoluble phosphates which gives a new avenue for the development of fungal biofertilizers after carrying out the necessary qualifying tests [24]. The *Aspergillus niger* solubilized maximum amount of tricalcium phosphate and from rock phosphate (385 μgmL^{-1}) at 15 days of incubation [25]. Similarly, mobilized phosphate observed between 320 μgmL^{-1} (*P. oxalicum*) and 500 μgmL^{-1} (*P. citrinum* and *P. purpurogenum*) from TCP at 15 days of incubation. The tricalcium phosphate was more efficiently solubilized than rock phosphate. The remarked in the poor solubilization of rock phosphate may be attributed to the complex mineral composition and particle size in the medium and in addition to the presence of strong apatite bond in the RP, which reduced phosphate solubilization [26].

Acidification by organic acid has been reported to be the main solubilization mechanism of inorganic P by microorganisms [27]. The decrease in pH values was recorded in all fungal isolates differently in the culture media. This might be due to the production of diverse organic acids from the available nutrient (glucose). The pH drop in cultures has been repeatedly reported by a number of research findings [28]. The pH values decreased to

variable levels depending on the culture type and later became nearly constant or increased with a reduction in mobilized phosphate. It is generally accepted that the major mechanism of mineral phosphate solubilization is due to the action of organic acids synthesized by soil microorganisms. Several authors have studied the ability of fungi, mainly of genus *Aspergillus* and *Penicillium*, to solubilize phosphates under *in vitro* conditions. *Aspergillus* and *Penicillium* genera were the most well-studied fungi used in bioleaching studies as they were found to be able to liberate considerable amounts of organic acids such as citric, oxalic and tartaric acids which were thought to be the main phosphate solubilizing tool [29]. The inoculation of P-solubilizing microorganisms is a promising technique because it can increase P availability in soils fertilized with rock phosphates [30].

The key mechanism associated with solubilization of mineral phosphates is the reduction of the pH of the medium by the release of low molecular weight organic acids by microorganisms [31]. These organic acids act removing inorganic P from soil particles of clay either by the direct exchange as chelation of metal ions in complex P-cations [32]. The release of anions also results in the rhizosphere acidification, directly increasing the solubility of inorganic precipitated salts of P. However, the soil microorganisms show wide variation in their ability to secrete organic acids and thus solubilize mineral phosphate [33]. There are various strategies have sought the use of microorganisms with potential for RP solubilization [34] to increase the availability of this nutrient from different types of phosphates of low solubility [35], reducing the cost and energy loss for the agronomic use of these sources of P [36]. The great advantage of this combined use, in addition to the exploration of alternative sources for P fertilization [37], is the use of rocks that have low levels of P, which are inadequate for the fertilizer industry because they contain a high degree of impurities, such as marginal rocks and wastes from industry.

CONCLUSION

The phosphatic fertilizer management is very important in the cash-crops like sunflower, cotton, chilly, tomato, black gram, sorghum, brinjal, green gram, Okra, and red gram. The applied phosphatic fertilizers have been chemically fixed by certain ions to form insoluble P form. The cost of the fertilizer as well as yield attributes has been gradually decreased. These problems can be easily rectified by application of phosphate solubilizing fungi as biofertilizer to the agricultural fields. The *in vitro* study revealed that the PSF is able to solubilize the various phosphates as in the form of Tricalcium phosphate (TCP) and Rock phosphate (RP).

By the application of PSF biofertilizer, can able to get the sustainable yield by dissolution of chemically fixed phosphorus.

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Table 1: Population dynamics of PSF

Sr. No.	Sample	Code Number	Population-level of PSF (x 10^4 g. soil dry wt.)
1.	Sunflower	SFF1 SFF2	2.11
2.	Cotton	CF1 CF2	1.52
3.	Chilly	CHF1 CHF2	0.64
4.	Tomato	TF1 TF2	5.95
5.	Black gram	BGF1 BGF2	0.62
6.	Sorghum	SF1 SF2	0.50
7.	Brinjal	BF1 BF2	2.20
8.	Green Gram	GGF1 GGF1	1.53
9.	Okra	OF1 OF2	1.09
10.	Red Gram	RGF1 RGF2	2.20

Table 2: Identification of Fungal Isolates

S. No.	Code No.	Fungi
1	SFF1	*Aspergillus niger*
2	SFF2	*Aspergillus flavour*
3	CF1	*Aspergillus niger*
4	CF2	*Penicillium notatum*
5	CHF 1	*Aspergillus niger*
6	CHF 2	*Penicillium notatum*
7	TF1	*Aspergillus flavus*
8	TF2	*Aspergillus flavus*
9	BGF1	*Aspergillus niger*
10	BGF2	*Aspergillus flavus*
11	SF1	*Aspergillus flavus*
12	SF2	*Aspergillus niger*
13	BF1	*Aspergillus flavus*
14	BF2	*Aspergillus flavus*
15	GGF1	*Aspergillus niger*
16	GGF2	*Aspergillus flavus*
17	OF1	*Aspergillus niger*
18	OF2	*Penicillium notatum*
19	RGF1	*Penicillium notatum*
20	RGF2	*Aspergillus flavus*

Table 3: Characterization of PSF with tricalcium (TCP) as P source

PSB Strains	Solubilization zone formation (mm)	pH Reduction	Organic production (0.1 N NaOH consumed)	Phosphatase enzyme (µmole/ml/hr)	Available Phosphorous (ppm)
SFF1	8.0	4.7	26.0	18.4	38.5
SFF2	7.0	4.9	24.5	21.7	25.5
CF1	7.0	4.7	16.6	15.3	20.0
CF2	6.0	4.6	15.0	13.5	23.5
CHF1	6.0	4.6	13.0	13.3	30.0
CHF2	7.0	4.5	25.6	23.7	37.5
TF1	7.0	4.1	27.8	24.6	29.5
TF2	6.0	4.4	15.5	12.6	28.0
BGF1	7.0	4.5	26.3	25.3	35.5
BGF2	6.0	4.3	26.5	25.1	28.5
SHF1	5.0	4.7	18.1	17.9	28.0
SHF2	5.0	4.8	16.4	16.4	35.0
BF1	6.0	4.7	14.3	14.4	39.5
BF2	5.0	4.6	17.0	16.2	32.5
GGF1	6.0	4.5	16.3	14.7	18.0
GGF2	8.0	4.0	29.7	28.8	50.0
OF1	6.0	4.6	10.2	11.8	19.5
OF2	5.0	4.8	11.5	12.3	28.0
RGF1	4.0	4.6	16.0	13.2	37.0
RGF2	6.0	4.2	26.7	26.7	42.0

Table 4: Characterization of PSF with rock phosphate (RP) as P source

PSB Strains	Solubilization zone formation (mm)	pH Reduction	Organic production (0.1 N NaOH consumed)	Phosphatase enzyme (µmole/ml/hr)	Available Phosphorous (ppm)
SFF1	5.0	4.9	13.2	19.5	27.5
SFF2	6.0	4.5	14.1	22.8	28.5
CF1	5.0	4.7	12.6	16.4	25.8
CF2	6.0	4.6	13.7	23.6	27.3
CHF1	6.0	4.5	14.3	24.4	27.5
CHF2	6.0	4.5	14.9	29.8	32.3
TF1	8.0	4.1	18.1	28.7	37.8
TF2	5.0	4.8	12.1	13.7	25.3
BGF1	6.0	4.7	16.1	26.4	34.5
BGF2	6.0	4.2	14.2	25.2	30.3
SHF1	5.0	4.9	12.6	17.0	23.0
SHF2	6.0	4.0	14.6	17.5	29.8
BF1	6.0	4.6	13.6	15.5	27.0
BF2	6.0	4.5	13.2	17.3	26.3
GGF1	6.0	4.6	16.1	25.8	33.8
GGF2	7.0	4.2	12.1	26.9	23.5
LF1	6.0	4.7	15.9	26.9	33.8
LF2	6.0	4.3	13.3	28.0	34.0
RGF1	6.0	4.5	16.2	27.3	36.3
RGF2	8.0	4.0	21.7	29.8	39.5