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Isolation Screening and Selection of Phosphate Solubilizing Fungi from Maize Rhizosphere

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A B S T R A C T

Laboratory experiments were conducted to isolate screen and select the efficient P-solubilizing fungal isolates from maize rhizosphere in the districts of Northern Karnataka. 21 P-solubilizing fungal isolates were obtained from the soil samples and were screened for phosphorus solubilizing ability on Pikovskaya's agar and broth M-10(1) showed highest phosphorus solubilizing activity. Five efficient isolates were selected and assessed for other functional activities such as zinc and potassium solubilization, phytohormone production (IAA and GA_3) and cellulolytic activity. All five isolates were able to solubilize zinc and produce phytohormones. Among the five isolates M-10(1) was highest in phytohormone production and DM-3(2) was the only isolate to produce cellulase enzyme.

Introduction

Phosphorus is a limiting nutrient in crop production. Therefore, it regulates the crop growth and yield to the greater extent thus its deficiency becomes an important chemical factor restricting plant growth in soils. Various factors can be responsible for phosphorus availability to crop plants. These include the form of native soil phosphorus, the type of phosphorus applied to the soil and reaction. As a result of strong adsorption of phosphate by iron and aluminum oxides, less than 1 per cent of soil phosphorus is available for plant uptake (Xavier *et al.,* 2011). Therefore, primary approach in management of phosphorus is to scavenge the native or fixed phosphorus and also to minimize the fixation of applied phosphorus fertilizer. The low cost practice to achieve this objective is to inoculate soil with the phosphorus solubilizing microorganisms.

Microbial solubilization of inorganic phosphate occurs by various mechanisms, such as acidification, chelation, ion exchange reactions and polymeric substances formation. There are two ways in microbial phosphorus solubilization, by solubilization processes and from P accumulation in the microbial biomass. The phosphorus solubilizing microorganisms

include fungi, bacteria and actinomycetes. Among these P-solubilizing microorganisms fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria. Since phosphorus solubilizing fungi does not lose the phosphorus dissolving activity upon repeated sub culturing under laboratory conditions as the phosphorus solubilizing bacteria do (Sperber, 1958; Kucey, 1983) and they produce more acids than bacteria (Venkateswarlu *et al.,* 1984). Moreover, fungi in soils are able to traverse long distances more easily than bacteria and hence, may be more important to phosphorus solubilization in soils (Kucey, 1983). Several soil fungi, particularly those belonging to the genera *Penicillium* and *Aspergillus* possess 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.

Materials and Methods

The present study was conducted at Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad. Objectives of the present study were to isolate, screen and select the phosphorus solubilizing fungi from different locations of northern Karnataka (Belagavi, Haveri, Dharwad districts). The soil samples were collected from maize rhizosphere for isolation of P-solubilizing fungi, covering three districts of Northern Karnataka and the geographical details of the study area are given in Table 1.

Isolation of P-solubilizing fungi from soil samples

Isolation of P-solubilizing fungi was carried out by serial dilution and pour plate technique. Pikovskaya's agar amended with tri-calcium phosphate was used to isolate the P-solubilizing fungi. The clear halo zone around the colonies was considered as positive result for P-solubilization.

Colony morphology of phosphorus solubilizing fungal isolates on solid media

The colour, type and shape of the phosphorus solubilizing fungal colonies were studied. The fungal isolates were grown on potato dextrose agar medium for one week at 28±2°C and the colony characters were recorded.

Screening of phosphorus solubilizing fungal isolates (PSF) by zone of solubilization and inorganic phosphate *(Pi)* released from tri-calcium phosphate

The phosphorus solubilizing fungal isolates were screened for their phosphorus solubilizing ability and inorganic phosphate release. The quantitative assay (zone of solubilization) was carried out for all the 21 phosphorus solubilizing fungal isolates by spotting 10 µl of spore suspension of the culture on sterile Pikovskaya's medium (Pikovskaya, 1948) and the zone of solubilization was calculated.

Inorganic phosphate (*Pi*) released from tri-calcium phosphate was estimated in pikovskaya's broth amended with tri-calcium phosphate. Cultures were grown in Pikovskaya's broth and the amount of *Pi* released in the broth was estimated at 5, 10 and 15 days after incubation in comparison with the un-inoculated control. The TCP broth cultures were spun at 10,000 rpm for 10 minutes to separate the cells and insoluble phosphate. The available phosphorus content in the supernatant was estimated by phosphomolybdic blue colour method (Jackson, 1973).

Selection and tentative identification of the phosphorus solubilizing fungal isolates

Based on the solubilization index and inorganic phosphorus release by the fungal isolates, five morphologically different, compatible and efficient phosphorus

solubilizing fungal isolates were selected and recoded for further experiment.

The isolates were tentatively identified up to generic level by observing the spore structure and arrangement in compound microscope using lactophenol blue.

Phytohormone production

The selected isolates were tested for production of phytohormones such as Indole 3-acetic acid and Gibberellic acid. The production of IAA was estimated by following the method given by Gordon and Paleg, (1957) and Gibberellic acid (GA_3) production was tested in Czapeck's broth containing 1 per cent glucose and peptone. The test organisms were inoculated in the broth. After 10 days of incubation the concentration of GA_3in culture broth was determined by spectrophotometric method using phosphomolybdic acid reagent (Deshmukh and Shinde, 2016).

Potassium solubilization

The selected PSF isolates were spotted on sterile Alexandrove's agar plates and incubated at 28±2°C for 72 hours. The isolates having potassium solubilizing ability showed clear zone around the colony.

Zinc solubilization

The selected PSF isolates were spotted on the sterile liquid mineral salt medium specified by Saravan *et al.,* (2003) and incubated at 28±2°C for 72 hours. The isolates having zinc solubilizing ability showed clear zone around the colony which indicated the solubilization of zinc in agar plates.

Cellulolytic activity

Fungal cultures were spotted on carboxy methyl cellulose agar media (CMC agar) and incubated at 28±2°C for 3 days. The plates were flooded with 0.1 per cent aqueous congo red and allowed for 30 min, washed with 1 ml NaCl (sodium chloride 1M) and allowed for 15 min. the colonies that hydrolyzed cellulose showed clear zones against red colour of non-hydrolyzed medium (Sicuia *et al.,* 2016).

Results and Discussion

Isolation and *In vitro* screening of phosphate solubilizing fungi (PSF)

A total of 47 rhizosphere soil samples were collected from three districts of Northern Karnataka (Haveri, Dharwad, Belagavi). The details such as location and geographical position of the site of soil sampling were taken (Table 1). Twenty one P-solubilizing fungal isolates were isolated from the collected soil samples (Table 2).The isolated fungal colonies were observed for colony morphology and the details are listed in Table 2. The isolates were assayed for *in vitro* phosphorus solubilization. The zone of solubilization, phosphate solubilization index and inorganic phosphate release by the isolates were assessed and the results are furnished in table 3.

Among all isolates M-10(1) showed maximum zone of solubilization and phosphate solubilization index (1.0 cm and 3.0) which was on par with M-9(5) (0.99 cm and 2.99) and BM-9(5) (0.99 cm and 2.99). However, the lowest was recorded by DM-2(2) (0.1 cm and 2.1).

The inorganic phosphate (*Pi*) release by the fungal isolates increased with incubation time and was highest at 15 days incubation time. The maximum *Pi* release was observed in M-10(1) (10.17 ppm) followed by BM-9(5) (9.91 ppm) and M-9(5) (9.86 ppm) at 15^{th} day. The lowest *Pi* release was observed in DM-2(2) (3.44 ppm). The *Pi* release in the broth at 15 days of incubation ranged from 10.17 to 3.44 ppm (Table 3).

Selection and tentative identification of the PSF isolates

Considering the phosphate solubilization and colony morphology, five morphologically different and biocompatible isolates with high phosphate solubilizing ability were selected for further study (M-10(1), M-9(5), BM-18(2), BM-9(5) and DM-3(2)). The selected PSF isolates were identified up to generic level based on their microscopic observation of spore structure and arrangement (Table 4).

In vitro synthesis of IAA and GA_3

The production of IAA by PSF isolates varied from 5.96 to 7.84 µg per ml of broth medium. Among all the isolates verified, M-10(1) produced high amount of IAA (7.84 µg/ml of medium) followed by M-9(5) (7.28µg/ml of medium). Lowest amount of IAA was synthesized by BM-18(2) (5.96 µg/ml of medium) (Table 4). However, the production of GA_3 by PSF isolates varied from 2.31 to 2.96 µg per 25 mlof broth medium. Among all the isolates verified, M-10(1) produced highest amount of GA_3 (2.96 µg/25 ml of medium) followed by M-9(5) (2.72µg/ml of medium). Lowest amount of GA_3 was synthesized by BM-18(2) (2.31 µg/25 ml of medium) (Table 4).

Potassium and zinc solubilization

Zinc and potassium solubilizing abilities of the PSF isolates were tested using ZnO mineral salt agar and Alexandrove's agar respectively. All the PSF isolates showed zinc solubilization, whereas none of them showed potassium solubilization (Table 4).

Cellulolytic activity of PSF isolates

All the selected PSF isolates were assessed for their cellulolytic activity on carboxy methyl cellulose agar (CMC) medium. Among all the isolates tested only DM-3(2) showed cellulose degradation (Table 4).

Fig.1 Inorganic phosphate (*Pi*) release by the phosphorus solubilizing fungal isolates in Pikovskaya's broth

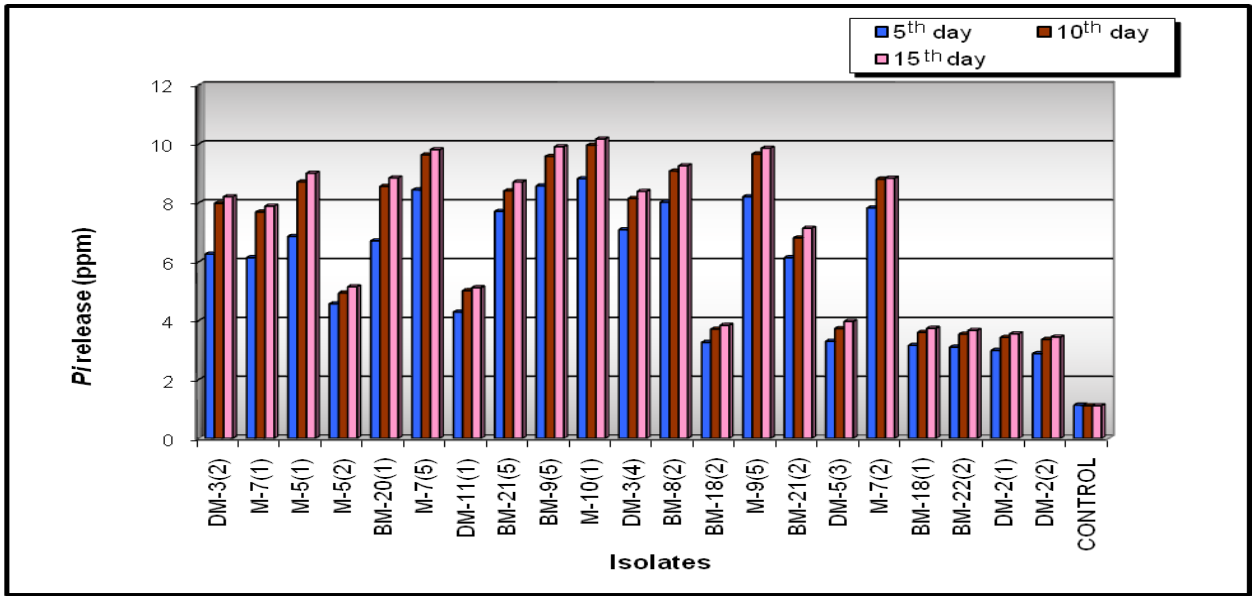


Table.1 Geographical positions of soil sampling sites

Sl. No.	Sample number	Location	Latitude (N)	Longitude (E)	Elevation (m)
Haveri					
01	M-1	Ranebennur	14°37' 20.7"	75°36' 29.1"	595
02	M-2	Hanumanamatti	14°39' 13.9"	75°34' 07.0"	598
03	M-3	Kakola	14°41' 06.5"	75°32' 21.5"	606
04	M-4	Motebennur	14°43' 38.3"	75°28' 14.2"	595
05	M-5	Haveri (Hanagal road)	14°47' 10.2"	75°23' 00.0"	575
06	M-6	Haveri	14°49' 44.4"	75°21' 50.0"	561
07	M-7	Punemanehalli	14°52' 53.6"	75°18' 32.7"	559
08	M-8	Bankapur	14°55' 44.3"	75°16' 06.7"	565
09	M-9	Bisnalli	14°57' 21.0"	75°14' 41.7"	596
10	M-10	Shiggaon	15°00' 22.8"	75°12' 05.1"	647
11	M-11	Kadahalli	15°03' 18.4"	75°10' 02.0"	688
12	M-12	Jigaluru	15°05' 40.2"	75°09' 08.7"	661
13	M-13	Varur	15°11' 59.9"	75°08' 11.2"	631
Belagavi					
01	BM-1	Kittur	15°40' 55.9"	74°54' 58.9"	682
02	BM-2	Timmapur	15°42' 29.6"	74°54' 54.3"	650
03	BM-3	Shigihalli	15°43' 47.7"	74°53' 10.5"	668
04	BM-4	Dastikoppa	15°48' 19.6"	74°51' 57.4"	657
05	BM-5	M K Hubli	15°50' 17.5"	74°52' 33.4"	676
06	BM-6	Hirebagewadi	15°56' 24.1"	74°57' 17.3"	682
07	BM-7	Halaga	15°54' 32.6"	74°49' 54.3"	745
08	BM-8	Mutenatti	15°54' 06.3"	74°47' 08.8"	761
09	BM-9	Hallabavi	15°55' 33.0"	74°46' 55.9"	760
10	BM-10	Hattaragi	15°56' 32.0"	74°47' 56.1"	736
11	BM-11	Gotur	15°56' 46.1"	74°48' 07.6"	748
12	BM-12	Hukkeri	16°00' 36.1"	74°49' 39.3"	553
13	BM-13	Ghataprabha	16°10' 51.9"	74°49' 53.0"	552
14	BM-14	Arabhavi	16°10' 56.8"	74°49' 52.7"	542
15	BM-15	Gokak	16°10' 52.2"	74°49' 51.4"	552
16	BM-16	Kolavi	16°12' 41.7"	74°46' 21.0"	561
17	BM-17	Khanagaon	16°14' 43.1"	74°41' 33.5"	621
18	BM-18	Nesaragi	16°14' 20.1"	74°37' 01.7"	671
19	BM-19	Harugoppa	16°09' 21.8"	74°32' 56.7"	675
20	BM-20	Murgod	16°05' 30.4"	74°31' 06.5"	686
21	BM-21	Bailhongal	15°57' 31.4"	74°30' 50.6"	743
22	BM-22	Belavadi	15°39' 47.6"	74°44' 02.5"	662

Table.1 Contd…..

Sl. No.	Sample number	Location	Latitude (N)	Longitude (E)	Elevation (m)
Dharwad					
01	DM-1	Kanavihonnapura	15°40' 56.1"	74°54' 58.2"	680
02	DM-2	Kanavihonnapura	15°22' 09.2"	75°00' 22.1"	687
03	DM-3	Jodahalli	15°20' 16.0"	75°00' 31.6"	617
04	DM-4	Hirehonnalli	15°13' 00.7"	75°00' 04.7"	543
05	DM-5	Harogera cross	15°13' 19.4"	75°01' 00.4"	576
06	DM-6	Kamadhenu cross	15°14' 00.3"	75°01' 35.4"	581
07	DM-7	Ukkinakere	15°15' 08.0"	75°02' 25.0"	574
08	DM-8	Kusugal	15°23' 19.6"	75°12' 24.8"	642
09	DM-9	Behatti	15°27' 12.5"	75°14' 33.1"	610
10	DM-10	Sulla	15°27' 04.9"	75°11' 56.4"	619
11	DM-11	Sulla	15°27' 12.9"	75°10' 41.2"	640
12	DM-12	Somanahalli	15°29' 22.6"	75°04' 42.0"	660

Table.2 Colony morphology of phosphorus solubilizing fungal (PSF) isolates on potato dextrose agar

Sl. No.	Isolates	Colony colour	Texture	Margin
01	DM-5(3)	Greenish white	Flat	Round, even
02	BM-18(1)	Black	Cottony	Round, even
03	DM-2(1)	Black	Flat	Round, even
04	DM-2(2)	White colony brown center	Flat, powdery	Round, uneven
05	BM-18(2)	White colony brown center	Flat, powdery	Round, uneven
06	BM-22(2)	Black	Flat	Round, even
07	M-7(2)	Bottle green with white mycelia	Raised	Round, uneven
08	M-5(1)	Black	Flat	Round, even
09	DM-3(4)	Bottle green with white mycelia	Raised	Round, uneven
10	BM-9(5)	Black	Flat	Round, even
11	M-10(1)	Black	Flat	Round, even
12	BM-20(1)	Black	Flat	Round, even
13	DM-3(2)	White colony brown center	Flat, powdery	Round, uneven
14	DM-11(1)	Black	Flat	Round, even
15	M-9(5)	Orange	Flat, powdery	Round, even
16	M-7(1)	Black	Flat	Round, even
17	BM-21(5)	Black	Flat	Round, even
18	M-7(5)	Black	Flat	Round, even
19	M-5(2)	Black	Flat	Round, even
20	BM-21(2)	Black	Flat	Round, even
21	BM-8(2)	Black	Flat	Round, even

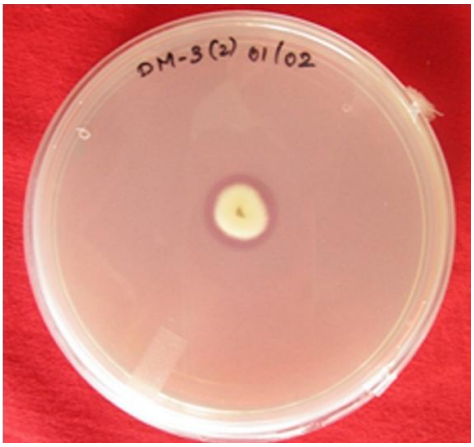
Table.3 Zone of solubilization and inorganic phosphate (*Pi*) release by the phosphorus solubilizing fungal isolates on Pikovskaya's agar and broth

Sl. No.	Isolates	Zone of solubilization (cm)	Solubilization index	*Pi* release (ppm)		
				5th day	**10th day**	**15th day**
01	DM-5(3)	0.41	2.41	3.30	3.73	3.97
02	BM-18(1)	0.26	2.26	3.26	3.71	3.84
03	DM-2(1)	0.14	2.14	2.99	3.43	3.55
04	DM-2(2)	0.10	2.10	2.88	3.36	3.44
05	BM-18(2)	0.40	2.40	3.26	3.71	3.84
06	BM-22(2)	0.12	2.12	3.10	3.54	3.67
07	M-7(2)	0.81	2.81	7.83	8.81	8.84
08	M-5(1)	0.93	2.93	6.86	8.71	9.01
09	DM-3(4)	0.79	2.79	7.09	8.15	8.39
10	BM-9(5)	0.99	2.99	8.58	9.58	9.91
11	M-10(1)	1.00	3.00	8.82	9.96	10.17
12	BM-20(1)	0.92	2.92	6.71	8.56	8.85
13	DM-3(2)	0.88	2.88	6.26	7.98	8.21
14	DM-11(1)	0.61	2.61	4.29	5.02	5.12
15	M-9(5)	0.99	2.99	8.21	9.66	9.86
16	M-7(1)	0.81	2.81	6.14	7.69	7.89
17	BM-21(5)	0.92	2.92	7.71	8.41	8.71
18	M-5(2)	0.67	2.67	4.57	4.94	5.15
19	M-7(5)	0.97	2.97	8.44	9.63	9.81
20	BM-21(2)	0.74	2.74	6.14	6.81	7.14
21	BM-8(2)	0.86	2.86	8.02	9.08	9.26
S. Em. ±		0.035	0.035	0.035	0.036	0.030
C. D. at 1 %		0.141	0.141	0.140	0.145	0.121

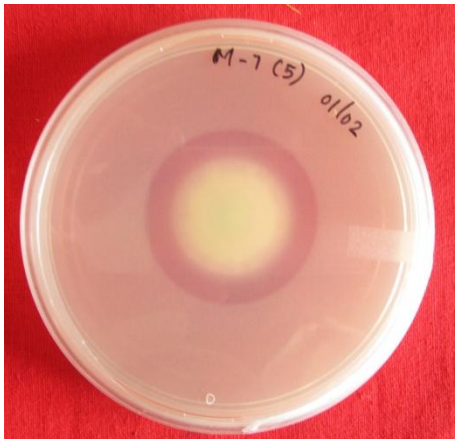
Table.4 Identification of selected PSF isolates up to generic level based on microscopic observation and their functional characterization

Sl. No.	Isolate no.	Probable Genus	Spore character	Potassium solubilization	Zinc solubilization	IAA (µg/ml)	GA (µg/25ml)	Cellulolytic activity
1	M-10(1)	*Aspergillus* sp.	Conidiophore terminated by swollen vesicle bearing flask shaped sterigmata which give rise to chain of conidia	-	+	7.84	2.96	-
2	M-9(5)	*Penicillium* sp.	Conidiophore terminated by cluster of flask shaped sterigmata which give rise to chain of conidia which give finger like appearance	-	+	7.28	2.72	-
3	BM-18(2)	*Penicillium* sp.	Conidiophore terminated by cluster of flask shaped sterigmata which give rise to chain of conidia which give finger like appearance	-	+	5.96	2.31	-
4	BM-9(5)	*Aspergillus* sp.	Conidiophore terminated by swollen vesicle bearing flask shaped sterigmata which give rise to chain of conidia	-	+	6.93	2.63	-
5	DM-3(2)	Unidentified	-	-	+	6.52	2.44	+

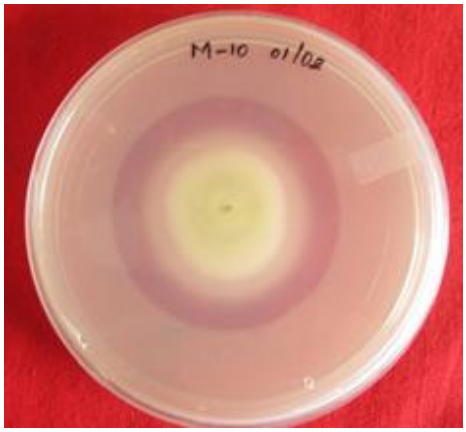
Plate.1 Phosphate solubilization by the fungal isolates on Pikovskaya's agar



DM-3(2)

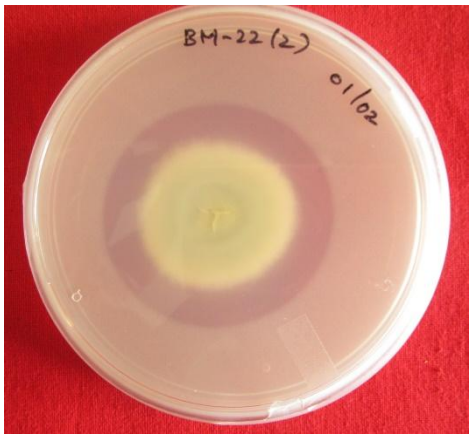


M-7(5)

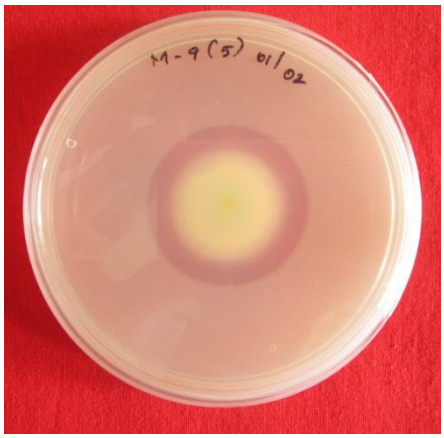


M-10(1)

DM-11(1)



BH-22(2)



M-9(5)

The soil samples were collected initially from rhizosphere of maize plant from three districts of Northern Karnataka (Haveri, Dharwad, Belagavi) for isolation of PSF. In this study, out of 47 samples 21 P- solubilizing fungal isolates were isolated. The typical colony characters of the isolated PSF that appeared on Pikovskaya's agar ranged from flat powdery to raised and the colour of the colonies were green, black, brown, orange and white. The margin of the colony was either even or uneven. The isolated PSF were purified and maintained. Among the 21 PSF isolates M-10(1) showed maximum zone of solubilization, phosphate solubilization index and *Pi* release in Pikovskaya's agar and broth respectively followed by M-9(5) and BM-9(5) indicating that they exhibit strong mechanisms for solubilization of phosphate than other isolates. The variation in P-solubilization could be due to the difference in the amount and type of the organic acids secreted which directly influence the P-solubilization (Fig. 1).

Five morphologically different and efficient P-solubilizing fungal isolates were selected and tentatively identified based on their colony morphology and spore structure. Two isolates were identified as *Aspergillus* sp. and two isolates as *Penicilluim* sp. The selected P-solubilizing isolates were assessed for functional activities such as zinc and potassium solubilization, production of growth promoting hormones (IAA and GA_3). All the isolates were able to solubilize zinc and produce growth promoting hormones such as IAA and GA_3.Among the isolates, M-10(1) was found to produce maximum amount of IAA and GA_3 which was 7.84 µg/ml and 2.96 µg/ml respectively. This property of the PSF isolates describes the importance of these microorganisms in plant growth which can in turn result in higher yield.

Cellulolytic activity is one of the very important traits for the better survival and ecological fitness of the organism in the rhizosphere (Boer *et al.,* 2005). Hence the ability of P-solubilizing fungal isolates for cellulose degradation was tested on CMC agar medium. Cellulolytic activity of the selected 5 PSF isolates was tested on CMC agar medium. DM-3(2) showed positive result for cellulose hydrolysis. The cellulose degradation is due to the production of cellulase enzyme by the isolate which convert complex sugar cellulose into simple sugars.

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