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Solubilization of inorganic phosphate by phosphate solubilizing fungi isolated from paddy of Vidarbha region

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Abstract
Fungal strains were recorded by using dilution plate methods on pikovskaya's medium from Soil samples were collected from different localities in paddy growing part of Vidarbha region, Maharashtra India. *Aspergillus* spp. the most important genera isolated during this investigation. Phosphate solubilizing fungi (PSF) were selected by formation of halo zone around fungal colony on (PVK) agar plate after 4 days of incubation. PSF were identified morphologically. All isolates exhibit different levels of phosphate solubilizing activity on (PVK) broth medium containing tricalcium phosphate (TCP) as sole phosphorus source. Final pH of the medium solubilize and recorded. *Aspergillus niger* and *Aspergillus flavus variable* and *Aspergillus niger*- 1 showed Solubilization potential or activity 4.77, 4.00 and 3.00 mm clear zone on 3.5 and 7 days after incubation respectively whereas the pH values of the culture filtrates were 3.50 and 3.10 after 8 and 15 days of incubation in pikovskayas broth medium respectively.

Keywords: Phosphate solubilizing fungi, aspergillus, halo zone

Introduction
Use of voluminous amounts of chemical fertilizers and fungicides has been an impediment to development of sustainable agriculture. Employment of bio fertilizers and bio pesticides may be able to side-step some of the deleterious effects caused by chemical fertilizers. Phosphorus (P) is one of the most important macro elements after nitrogen for both plant and microorganisms. It is structural and functional element as well as energy transfer.
In general, Indian soils have low phosphorus appearance that cannot cover the demands of plant. The Application of chemical fertilizers to the soil is not successful pathway because it is rapidly fixed Where it react with iron, aluminum and manganese in acid soil and with calcium in neutral and alkaline. Soil and precipitated strongly to the surface of the soil particles, this reaction remove available phosphorus from soil solution and become unavailable to plant. Microorganisms are involved in a range of processes that affect the transformation of soil 'P' and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization. Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturist as soil inoculums to improve the plant growth and yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by phosphate solubilizing fungi. These phosphate solubilizing fungi may vary in their functional diversity at various geographic locations and therefore, the present investigation was planned to isolate and characterize the phosphate solubilizing fungi from rhizosphere soils of different paddy growing regions of Vidarbha (Maharashtra).

Material and Methods
Soil sampling and isolation of phosphate solubilizing fungi
A total of 82 rhizosphere soil samples (0-15 cm depth) were collected from paddy growing regions of Vidarbha (Maharashtra). The isolation of phosphate solubilizing fungi was carried out on Pikovskaya's agar medium by serial dilution of soil and agar plating method.

Morphological characterization
The phosphate solubilizing fungal isolates were identified on the basis of cultural and microscopic features. The colonial morphology of fungal isolates was examined on Pikovskaya's agar medium and microscopic appearance by lacto phenol cotton blue staining technique which determined the type of reproductive mycelium i.e. conidiophores.

Phosphate solubilizing ability of the fungal isolates: The ability of the fungal isolates to solubilize insoluble inorganic phosphate was carried out by allowing the fungi to grow on selective media, i.e. Pikovskaya's agar plates and incubating at 28-30 °C for 3,5 and 7 days. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solubilizing activity of the fungus. The diameter of the zone of TCP solubilization was measured and expressed in millimeters. Quantitative estimation of Pi released from tricalcium phosphate for fungal isolates: The fungal isolates positive for P solubilization on Pikovskaya's agar medium were subjected to quantification of Pi released from TCP in broth medium. The Erlenmeyer flasks containing 50 ml Pikovskaya's broth were inoculated with 5 mm mycelial discs from 7 day old culture of each fungal isolate grown on PDA in three replicates and incubated for 8 and 15 days at 28 2 °C. The pH of the medium was adjusted to 7.0. The mycelia were harvested after 15 days of incubation by filtering and the reduction in the pH of the culture filtrate was recorded after centrifuging the medium at 10000 rpm for 5 min. so as to monitor the change in pH and study its correlation with the Pi released. The available P content of the supernatant was estimated by using phosphomolybdic blue colour method.

Morphological characterization

A distinct variation was observed in fungal and spore morphology among the phosphate solubilizing *Aspergillus* isolates (Table 1). On the basis of fungal and spore morphology, three distinct groups of *Aspergillus* isolates were formed and these were: 8 of the *Aspergillus* isolates produced white floccose mycelium; 7 with velvety green mycelium and 14 with white cottony loose woven thread like mycelium forming black-brown spores in due course. Based on colony morphology, spore characteristics and microscopic examination, out of 20 phosphate solubilizing fungal isolates, 18 were identified as *Aspergillus Niger* and 06 as *Aspergillus flavus*. These results are in agreement with the results of Mittal *et al*. who characterized phosphate solubilizing fungal strains up to species level based on colonial morphology, spore characteristics and microscopic examination and identified as *Aspergillus awamori and Penicillium digitatum.* Phosphate solubilizing ability of the fungal isolates: All the 24 *Aspergillus* isolate were tested for their ability to solubilize inorganic phosphate both qualitatively and quantitatively and their results are presented in table 2 and 3 respectively.

Quick analysis of P solubilization was carried out on Pikovskaya's agar medium. Among all the 07 isolates along with PDKV commercial strain were able to form zone of P-solubilization on the medium. The diameter of the zone of P solubilization ranged from 11 mm (A.N-1) to 30 mm (A.N-4) and 9 mm (A.f -) to 24 mm (PSF-61) for Aspergillus isolates respectively.

Quantitative estimation of Pi released from TCP

The studies on quantitative estimation of Pi released in Pikovskaya's broth showed that the *Aspergillus* isolates released 24.20 to 59.00 P_2O_5mg/100ml TCP after 8 and 15 days of incubation. Out of 20 Aspergillus isolates, the isolate (*A.n* -1) recorded significantly highest P-solubilization (59.00 P_2O_5mg/100 ml) followed by a.f-2, a.n-9 and a.n-11 with 54.00 54.00 and 39.00 P_2O_5mg/100ml Pi released respectively.

Among the 20 strains, *Aspergillus flavus*-1 were the most efficient solubilizing 59.00 P_2O_5mg/ 100ml total P in TCP broth. Decrease in pH of medium during phosphate solubilization: The decrease in pH of TCP broth from initially adjusted pH of 7.0 was also noted at 8 and 14 days after inoculation for Aspergillus isolates. Among Aspergillus isolates, the significant reduction in pH (3.10) was recorded by A.N-2 isolate followed by A.N-1, PSF-55 and PSF-64 isolates which reduced the pH of the medium to 3.54, 3.15 and 3.30 respectively (Table -). Pradhan and Sukla also reported positive correlation between amounts of 'P' solubilized with decrease in pH of the medium.

Results and Discussion

A total of forty-one (41) phosphate solubilizing Fungi were isolated from the eighty two (82) rhizospheric sample of rice plant of Vidarbha region, Maharashtra. Among them, 06 (Six) isolates show remarkable zone of solubilisation and these selected for morphological and other study. Studies on clear zone formation by selected PSF isolates around their colony on Pikovskaya's agar medium were also carried out in present investigation.

Identification of fungal isolates was done by observing colony characteristics on PDA plates. On the basis of growth pattern the isolates were identified as *Aspergillus* spp. This was confirmed by microscopic analysis of colony using lacto phenol blue stain. *Aspergillus* gave black dense felt like mycelial growth on front side of PDA plate with dirty white color on back side Figure 1. Microscopically, branched conidiophores and phialides were observed giving brush-like appearance. Conidia were globular, greenish and smooth.

Assessment of phosphate solubilizing activity of fungi by halo zone method.

Observations of fungal isolates were recorded at 3rd, 5th and 7th day. The data presented in Table 2 showed that on 3rd day of incubation fungal isolates formed clear halo zone around their colony. Isolates *i.e*. A. *flavus*-2, *A. niger*-1, *A. niger*-4 *A. niger*-9 and *A. niger*-11 showed maximum clear halo zone *i.e*. 5.00 mm, 3.00 mm, 2.68 mm, 3.00 mm and 2.66 mm with 27.00 mm, 22.33 mm, 23.67 mm, 18.00 mm and 15.66 mm total zone and 22.00 mm, 20.00 mm, 19.67 mm, 13.00 mm and 15.00 mm colony diameter respectively, followed by *A. niger-2* and *A. niger*-10 which formed 3.67 mm and 2.66 mm clear zone with 17.81 mm and 19.67 mm total zone and 14.00 mm and 17.00 mm colony diameter respectively while minimum clear zone *i.e*. 1.00 mm with 8.66 mm total zone and 7.66 mm colony diameter recorded by *A. flavus*- 4 (Plate 2a).

On 5th day of incubation *A. flavus-2, A. niger*-1, *A. niger*-4, *A. niger*-9 and *A. niger*-11 showed maximum halo zone of solubilization *i.e*. 8.00 mm, 4.00 mm, 3.66 mm, 4.00 mm and 3.66 mm with 33.00 mm, 48.00 mm, 46.66 mm, 33.00 mm and 28.00 mm of total zone and 25.00 mm, 44.00 mm, 43.00, 29.00 mm and 25.00 mm colony diameter respectively. While A. *flavus*-4 formed minimum halo zone *i.e*. 2.00 mm with 12.33 mm total zone and 10.33 mm colony diameter respectively.

On 7th day of incubation *A. flavus*-9 and A. *niger*-4 showed maximum solubilization clear halo zone *i.e*. 9.00 mm and 4.77 mm with 70.00 mm and 48.00 mm total zone and 39.00 mm and 65.33 mm colony diameter respectively, whereas minimum halo zone *i.e*.1.66 mm with 19.66 mm total zone and 17.33 mm colony diameter was recorded in *A. flavus*-3 and remaining fungal isolates showed halo zone ranged from 2.66 to 4.47 mm on Pikovskaya's agar medium.

This result indicate that those fungal isolates formed maximum halo zone on Pikovskaya's agar medium these isolates were effective phosphate solubilizers which confirmed the finding of Wani and Patil (1979) [6]. Padmawathi Tallapragada and Seshachala (2010) [7] who reported that *Aspergillus* spp. formed 26.00 mm halo zone and *Bacillus subtilis* produce 46.10 mm clear zone on Pikovskaya's agar medium.

Quantitative estimation of Pi released from TCP

The fungi isolates were tested for phosphate solubilizing activity *in vitro*. Test organisms were inoculated in Pikovskaya's broth medium containing tricalcium phosphate (0.5 g/100 ml) and incubated for 8th and 15th days at 28±2 °C and observations were recorded for reduction in pH and amount of solubilized phosphate (P_2O_5 mg/ml) in broth medium. Initial pH of the broth was 7.00 before inoculation.

The data presented in Table 3 revealed that after 8th day of inoculation with different fungal isolates the change reduction in pH of medium ranged from 7.00 to 3.48 and amount of P_2O_5 solubilized measured between 27.10 to 59.00 P_2O_5 mg/ml was observed, the pH of the medium was decreased due to production of organic acids in the medium by the isolates. Among all *Aspergillus* spp. maximum reduction in pH recorded in *Aspergillus niger*-1 *i.e.* 3.48 with maximum P_2O_5 solubilization *i.e.*45.00 P_2O_5 mg/ml followed by *Aspergillus niger*-4 (3.50 pH) with 41.80 P_2O_5 mg/ml, *Aspergillus niger*-9 (3.60 pH) 30.20 P_2O_5 mg/ml and *Aspergillus niger*-11 (3.83 pH) with 27.10 P_2O_5 mg/100 ml, whereas minimum reduction in pH recorded in *Aspergillus niger* - 5 (3.90 pH) with 24.20 P_2O_5 mg/100 ml as compare to control (6.80 pH) with 00.0 P_2O_5 mg/ ml. Other isolate of *Aspergillus flavus* -2 recorded reduction in pH 3.80 with 24.20 P_2O_5 mg/100 ml solubilization.

After 15th day of incubation *Aspergillus niger*-1 exhibited maximum reduction in pH *i.e.* 3.15 with maximum P_2O_5 solubilization *i.e.* 59.00 P_2O_5 mg/ml followed by *Aspergillus niger*-4 (3.10 pH) with 54.00 P_2O_5 mg/100ml, while *Aspergillus niger*-5 showed minimum reduction in pH (3.68) with minimum P_2O_5 solubilization *i.e.* 31.23 P_2O_5mg/100ml as compare to control (6.57 pH) with 2.00 P_2O_5 mg/100ml. *Aspergillus niger*-1 and *Aspergillus niger*-4 recorded high organic acid production, maximum reduction in pH and maximum P_2O_5 solubilization in Pikovskaya's broth medium on 8 th and 15 th day of incubation.

Similar findings reported by Alam *et al.* (2002) [1] and Rashid *et al.* (2004) reported maximum solubilization of tricalcium phosphate by PSM the range 0.04 to 0.147%. Among fungal isolates *Aspergillus Niger* showed maximum solubilization *i.e.* (0.45%) P_2O_5 in broth Manivannan *et al.* (2011) [3], Patil (2002) [4] and Chakraborty *et al.* (2010) [5] reported that *Aspergillus* spp. and *Penicillium* spp. solubilized maximum tricalcium phosphate ranged 799 to 856 mg/l in Pikovskaya's broth medium due to organic acid production.

Estimation of pH and phosphate solubilization by different fungal isolates in Pikovskaya's broth medium

The data presented in Table 3 revealed that on 8th day of incubation PSF isolates produce organic acid which decreased the pH of the medium and solubilized tricalcium phosphate in broth medium. Among all PSF isolates Maximum reduction in pH recorded in *Aspergillus Niger* -1 *i.e.* 3.80 with 41.00 P_2O_5 mg/100ml solubilization. Followed by *Aspergillus niger*-4 (4.00 pH) with 38.00 P_2O_5 mg/100 ml and minimum reduction in pH was recorded in *Aspergillus niger*- 5 *i.e.* 4.80 pH with 18.90 P_2O_5 mg/100ml as compare to control (6.90 pH) and 0.00 P_2O_5 mg/100 ml. as well as On 15th day of incubation, *Aspergillus niger* -1 *i.e.* 3.80 with 41.00 P_2O_5 mg/100ml solubilization. Followed by *Aspergillus niger*- 4 (4.00 pH) with 38.00 P_2O_5 mg/100 ml and minimum reduction in pH was recorded in *Aspergillus niger*- 5 *i.e.* 4.80 pH with 18.90 P_2O_5 mg/100 ml as compare to control (6.90 pH) and 0.00 P_2O_5 mg/100 ml P solubilization as compare to control. Among all PSF isolates *Aspergillus Niger* -1 and *Aspergillus niger*- 4 were efficient phosphate solubilizers.

Estimation of change in titrable acidity and pH by different phosphate solubilizing fungal isolates by titration method

Isolated phosphate solubilizing fungi were tested to measuring their change in pH and titrable acidity by titration method *in vitro*. The Pikovskaya's broth medium was inoculated with test organism and incubated for 8 th day at 28±2 °C and observations were recorded for change in titrable acidity and reduction in pH in broth medium. Initial pH of the broth medium was recorded at 7.00 before inoculation.

Data presented in Table 4 revealed that on 8th day of incubation all the fungal isolates produce various amount of organic acid. Among fungi *Aspergillus niger*-1 recorded maximum titrable acidity *i.e.* 3.40 ml with reduction in pH *i.e.* 3.48 as compare to control, followed by *A.niger*-4 (3.13 ml) with reduction in pH (3.50) and *A. niger* -9 (2.80 ml) with pH (3.60), whereas *A.niger*-5 showed minimum titrable acidity *i.e.* 2.49 ml with pH 3.90. The result showed that all the fungal isolates produce organic acid with decreased in pH.

The similar results were reported by Patil (2002) [4] reported that *Aspergillus flavus*, *Aspergillus niger* and *Penicillium* spp. produced organic acid and reduced the pH of the medium with P_2O_5 solubilization ranged from 65.40 to 137.4 P_2O_5 mg/100 ml. Alam *et al.* (2002) [1] observed that *Aspergillus* and *Penicillium* produce organic acid which reduced the pH of medium and solubilized tricalcium phosphate (0.22%) in liquid medium on 7th day of incubation.

Table 1: Fungal and spore morphology of *Aspergillus* and *Penicillium* isolates

S. N.	Fungal morphology	Spore morphology	Isolate number	Region of occurrence
	***Aspergillus* isolates**			
1.	White floccose mycelium	Black spores	7 isolates	Districts of Bhandara, Gadchiroli, Gondia
2.	Velvety green mycelium	Black spores	9 isolates	Districts of Chandrapur, Gadchiroli, Nagpur
3.	White cottony, loose woven thread like mycelium	Brown-black spores	4 isolates	Districts of Bhandara, Nagpur, Gondia

Table 2: Measurement of clear zone formation by fungal isolates around their colony on Pikovskaya's agar medium.

Sr. No	Isolates	3rd Day of Inoculation			5th Day of Inoculation			7th Day of Inoculation		
		TZ (mm)	CD (mm)	CZ (mm)	TZ (mm)	CD (mm)	CZ (mm)	TZ (mm)	CD (mm)	CZ (mm)
1	*A. niger*- 1	22.33	19.67	2.68	46.66	43.00	3.66	69.77	65.33	4.44
2	*A. niger*- 2	17.81	14.00	3.67	32.66	29.66	3.00	58.00	54.60	4.00
3	*A. niger*- 3	21.00	19.00	2.00	47.33	44.33	3.00	68.00	65.30	3.30
4	*A. niger*- 4	23.67	20.00	3.00	48.00	44.00	4.00	70.00	65.33	4.77
5	*A. niger*- 5	23.89	21.33	2.33	47.33	44.33	3.00	70.33	66.33	3.00
6	*A. niger*- 6	15.03	12.67	2.33	29.00	26.00	3.00	42.66	39.00	3.66
7	*A. niger*- 7	16.00	13.67	2.33	30.00	27.33	2.60	44.00	41.00	3.00
8	*A. niger*- 8	21.66	19.00	2.66	42.00	39.00	3.00	62.66	59.00	3.66
9	*A. niger*- 9	18.00	15.00	3.00	33.00	29.00	4.00	48.60	44.00	4.60
10	*A. niger*- 10	19.67	17.00	2.66	36.00	33.33	3.33	55.66	51.00	4.00
11	*A. niger*- 11	15.66	13.00	2.66	28.00	25.00	3.66	44.33	39.00	4.33
12	*A. niger*- 12	16.00	18.00	2.00	30.00	27.33	2.67	42.33	38.66	3.67
13	*A. niger*- 13	17.00	15.02	2.00	33.00	30.00	3.00	46.00	42.33	3.66
14	*A. niger*- 14	15.33	13.66	1.67	29.77	27.44	2.33	41.00	38.00	3.00
15	*A. niger*- 15	14.67	12.33	2.33	25.66	23.00	2.67	34.00	31.00	3.00
16	*A. niger*- 16	17.00	15.00	2.00	28.00	25.33	2.66	36.66	33.33	3.33
17	*A. flavus* –1	14.33	8.33	6.00	19.00	11.00	8.00	28.00	19.00	3.37
18	*A. flavus* –2	27.00	22.00	5.00	33.00	25.00	8.00	48.00	39.00	9.00
19	*A. flavus* –3	9.66	8.33	1.33	12.66	11.00	1.66	19.66	17.33	2.33
20	*A. flavus* – 4	8.66	7.66	1.00	12.33	10.33	2.00	18.66	16.00	2.67
	'F'- Test	Sig	Sig	Sig	Sig	Sig	Sig	Sig	Sig	Sig
	S. E. (m)±	0.66	0.71	0.38	1.04	0.98	0.21	0.97	0.78	0.25
	C.D.(p=0.01)	2.49	2.73	1.45	3.95	3.73	0.81	3.72	2.97	0.97

TZ = Total Zone, CD = Colony Diameter, CZ = Clear Zone

Table 3: Estimation of pH and phosphate solubilization by different fungal isolates in Pikovskaya's broth medium Initial pH 7

Sr. No.	P solubilizing fungal Isolates	8th day of incubation		15th day of incubation	
		Ph	P_2O_5 mg/100 ml	pH	P_2O_5 mg/ 100 ml
1	*Aspergillus niger*- 1	3.48	45.00	3.15	59.00
2	*Aspergillus niger*- 4	3.50	41.80	3.10	54.00
3	*Aspergillus niger*- 9	3.60	30.20	3.30	39.00
4	*Aspergillus niger*- 11	3.83	27.10	3.55	35.00
5	*Aspergillus niger*- 5	3.90	24.20	3.68	31.23
6	*Aspergillus flavus*- 2	3.80	30.00	3.54	54.00
7	Control	6.80	0.00	6.57	2.00
	'F'- Test	Sig	Sig	Sig	Sig
	S. E. (m)±	0.22	0.24	0.21	0.25
	C.D.(p=0.01)	0.81	0.93	0.78	0.95

Table 4: Estimation of change in titrable acidity and pH by 'P' solubilizing fungal Isolates

Sr. No.	'P' solubilizing fungal Isolates	8th day of incubation	
		Titrable acidity (ml)	pH
1	*Aspergillus niger*- 1	3.40	3.48
2	*Aspergillus niger*- 4	3.13	3.50
3	*Aspergillus niger*- 9	2.80	3.60
4	*Aspergillus niger*- 11	2.66	3.83
5	*Aspergillus niger*- 5	2.49	3.90
6	*Aspergillus flavus*- 2	2.70	3.80
7	Control	0.00	6.80
	'F'- Test	Sig	Sig
	SE(M) ±	0.25	0.24
	CD,(p = 0.01)	0.94	0.81

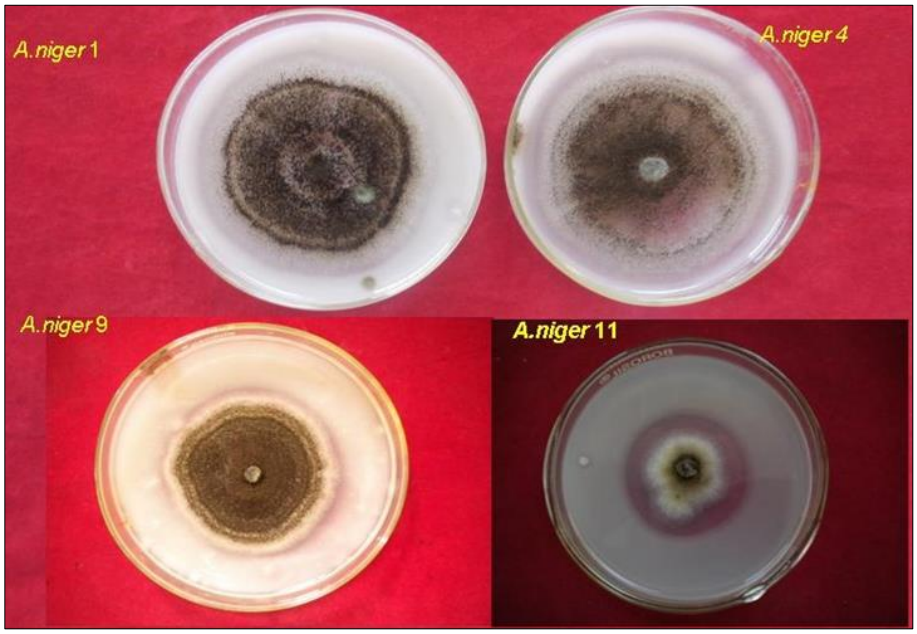

Fig 1: (A) Clear halo zone formed by phosphate solubilizing fungi

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Conclusion
In the present investigation, Paddy rhizospheric soils are rich in phosphate solubilizing Fungi. The isolate *Aspergillus niger*-4 on the basis of qualitative and quantitative estimation of phosphate solubilization showed maximum phosphate solubilizing activity. This strain can be used in the field as efficient bio fertilizers. Bio fertilizers are environment friendly, free from hazardous chemicals, possess no detrimental health effects and are cost effective.

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