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ORIGINAL ARTICLE

Effect of Mineral Phosphate Solubilization on Biological Nitrogen Fixation by Diazotrophic Cyanobacteria

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Abstract The ability of two diazotrophic cyanobacteria *Westiellopsis prolifica* and *Anabaena variabilis* were examined to solubilize extracellular insoluble tricalcium phosphate (TCP) and Mussorie rock phosphate (MRP). The two strains exhibited a differential response to insoluble forms of phosphorus used. *W. prolifica* showed better growth in presence of MRP while *A. variabilis* proliferated better in presence of TCP. Biological nitrogen fixation measured in terms of acetylene reduction (AR) activity showed significant variation among the concentrations of TCP or MRP and time of incubation. *W. prolifica* and *A. variabilis* showed maximum AR activity on 14 and 21 days of incubation respectively. In general AR activity in presence of MRP was always less than that in presence of TCP at all concentrations. Among the two cyanobacteria *A. variabilis* was best in terms of P-solubilization and nitrogen fixation and TCP (20 mg P l^{-1}) was the best source of insoluble P rather than MRP or K_2HPO_4.

Keywords AR activity · Diazotrophic cyanobacteria · Mussorie rock phosphate · Tricalcium phosphate · Available P

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Introduction

Phosphorus (P) is second only to nitrogen as an essential mineral fertilizer for crop production, comprising ~0.2% of plant dry weight. Further, soluble P is often the limiting mineral nutrient in soil and is conditioned by various factors. At any given time, a substantial component of soil P is in the form of poorly soluble mineral phosphates. These mineral phosphates are, in general, are biologically not available for nutritional transport and assimilation.

Cyanobacteria constitute the largest, most diverse and widely distributed group of prokaryotes that perform oxygenic photosynthesis and improve soil health [1]. The practical utility of these organisms as a source of biologically fixed nitrogen in paddies has been well recognized [2, 3]. Although, few reports exist on their ability to solubilize mineral P, not much attention has been paid to this attribute of these diazotrophs. They have been shown to solubilize insoluble $Ca_3(PO_4)_2$, $FePO_4$, $AlPO_4$, hydroxyapatite ($Ca_5(PO_4)_3{\cdot}OH$) and rock phosphate [4–6] in soils, sediments or in pure cultures. They are also known to solubilize organic sources of P [7].

The present investigation was carried to evaluate the efficiency of most common dinitrogen fixing cyanobacteria, *Westiellopsis prolifica* and *Anabaena variabilis* in presence of insoluble phosphate sources.

Materials and Methods

The mineral phosphates namely, Mussorie rock phosphate (MRP) was obtained from Pyrites and Phosphates Ltd., Dehradun, India and tricalcium phosphate (TCP) was procured from HiMedia Laboratories, Mumbai, India. The total P content in MRP and TCP was 18 and 20% respectively.

Unialgal cultures of heterocystous blue green algae (BGA) namely *W. prolifica* and *A. variabilis* were obtained from the germplasm of Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute (IARI), New Delhi and maintained and grown in N-free BG-11 medium [8] at 28 ± 2°C with 16/8 h L/D cycles at 2500–3000 Lux light intensity emanating from cool white fluorescent tubes. Their identification based on the microscopic morphological characters was confirmed using identification keys using standard literatures [9].

The experimental flasks were inoculated with 2% inoculum of P-starved, 10 day old actively growing cultures of both the algae for 35 days. The P-starved cultures were grown in presence of alternate P sources MRP and TCP at varying concentrations (equivalent to 10, 20 and 30 mg P l^{-1} by replacing K_2HPO_4 in the conventional BG-11 medium). Potassium chloride of equivalent amount (34.2 mg l^{-1}) was added in the medium to maintain the availability of potassium. Conventional BG-11 medium with K_2HPO_4 as source of P was maintained as control. The experiments were conducted in triplicates by taking 100 ml medium in 250 ml conical Erlenmeyer flasks. Growth as total chlorophyll content, nitrogen fixation and P-solubilization was measured periodically at 7 days interval up to 35 days. The growth of the cyanobacterial strains was estimated as total chlorophyll content by the Mackinney method [10]. Nitrogenase activity was estimated by the Acetylene Reduction (AR) activity as described by Hardy et al. [11]. The available 'P' in cell free supernatant was determined by vanadomolybdi-phosphoric yellow complex method [12, 13]. The number of heterocysts was counted in at least ten randomly selected filaments and the ratio of heterocysts to total number of cells was expressed as percentage.

Statistical Analysis

The data was subjected to analysis of variance (ANOVA) using the software MSTAT-C. Differences were considered to be significant at the 95% confidence level.

Results

The conventional source of P K_2HPO_4 in BG-11 medium was replaced with varying concentrations (10, 20 and 30 mg P l^{-1}) of MRP or TCP. Two strains of cyanobacteria, *A. variabilis* and *W. prolifica*, were evaluated for their ability to grow, solubilize P and fix nitrogen in presence of available form of P (K_2HPO_4) and insoluble sources of P (MRP and TCP). In general the growth of both the strains was significantly less in presence of either MRP or TCP as compared to K_2HPO_4. The two strains exhibited a differential response to insoluble forms of P used. *W. prolifica* showed better growth in presence of MRP while *A. variabilis* proliferated better in presence of TCP (Table 1). Among the concentrations of MRP and TCP used, maximum growth was exhibited at 20 mg TCP.

Variations were observed with regards to the ability to solubilize P by two cyanobacterial strains at different concentration of TCP and MRP. Available 'P' content in cultural filtrates showed that most of the total 'P' was in the available form (~80%) when K_2HPO_4 was used as a source of 'P' (Table 2). The amount of available 'P' in the culture filtrates of the organisms, when 'P' was supplied as an insoluble source (MRP or TCP), was very less as compared to total 'P' content and also, the availability was concentration dependent (10 mg < 20 mg < 30 mg 'P'). The available 'P' followed a definite pattern and decreased with incubation time irrespective of its concentration or source. The maximum availability of P was shown during early stages of incubation which gradually decreased with time of incubation in both the organisms. It reduced drastically (>60%) at lower concentration (10 mg P l^{-1}) of MRP but the reduction was 30–40% at higher concentrations (20 and 30 mg P l^{-1}) by the end of incubation. Tricalcium phosphate was solubilized more effectively by both the algae resulting in more available 'P' throughout the experimental phase as well as at the end of incubation.

The two cyanobacterial strains responded differently for nitrogen fixation in presence of K_2HPO_4, TCP and MRP. AR activity values showed significant variation among the concentrations of TCP or MRP and time of incubation. *W. prolifica* and *A. variabilis* showed maximum AR activity on 14 and 21 days of incubation respectively. Both the strains showed maximum AR activity in presence of 20 mg 'P' (TCP) after 21 days of incubation. In general AR activity in presence of MRP was always less than that in presence of TCP at all concentrations. The heterocyst frequency was calculated at regular intervals in both cultures showed a gradual increase in frequency (Table 3).

Discussion

Cyanobacteria are used as biofertilizer mainly because of their ability to fix atmospheric nitrogen. However there are reports available on the ability of cyanobacteria to solubilize P [15]. In the present study, *W. prolifica* and A. *variabilis* was found to exhibit growth and solubilize P when grown in presence of MRP or TCP. Roychoudhury and Kaushik [4] have reported that MRP supported luxuriant growth of cyanobacteria and biomass of some cyanobacteria was more in media containing MRP compared to medium containing K_2HPO_4, but on the other hand the biomass of *Tolypothrix* in presence of K_2HPO_4 was more

Table 1 Time course effect of different phosphate sources on total chlorophyll* content in cyanobacteria

Treatments(T)		Control (-P)	K_2HPO_4	MRP (mg P l^{-1})			TCP (mg P l^{-1})			Mean (SXD)	Mean (S)
Strains (S)	Days (D)			10	20	30	10	20	30		
Westiellopsis prolifica	7	0.05	0.09	0.04	0.04	0.02	0.05	0.06	0.07	0.05	0.75
	14	0.27	0.71	0.27	0.40	0.20	0.39	0.35	0.20	0.35	
	21	0.30	3.63	0.60	0.82	0.44	0.54	0.70	0.38	1.04	
	28	0.13	1.99	1.15	0.14	0.80	0.97	1.32	0.86	0.92	
	35	0.15	1.73	1.47	1.73	1.22	1.63	1.61	1.61	1.39	
Mean (SXT)		0.18	1.63	0.71	0.63	0.52	0.72	1.00	0.62		
Anabaena variabilis	7	0.02	0.03	0.04	0.05	0.03	0.08	0.04	0.05	0.04	0.93
	14	0.19	1.24	0.18	0.31	0.22	0.43	0.67	0.59	0.48	
	21	0.13	1.99	0.48	0.55	0.38	1.15	1.66	1.41	0.97	
	28	0.13	2.26	1.11	1.21	0.67	1.36	1.88	1.87	1.31	
	35	0.12	4.67	1.23	1.48	0.98	1.61	2.24	2.45	1.85	
Mean (SXT)		0.12	2.04	0.61	0.72	0.46	0.93	1.30	1.27		
TXD	7	0.04	0.06	0.04	0.05	0.03	0.07	0.05	0.06		
	14	0.23	0.98	0.23	0.36	0.21	0.41	0.51	0.40		
	21	0.22	2.81	0.54	0.69	0.38	0.85	1.66	0.90		
	28	0.13	2.13	1.13	0.68	0.74	1.17	1.60	1.37		
	35	0.14	3.20	1.35	1.61	1.10	1.62	1.93	2.03		

	S	TXS	TXD	SXD	TXSXD
SEM±	0.0048	0.0135	0.0213	0.0107	0.0302
CD ($P = 0.05$)	0.0133	0.0374	0.0590	0.0296	0.0837

*Values are μg chlorophyll ml^{-1}

than in MRP. This suggests that the organisms vary in their capacity to grow in insoluble inorganic P sources like MRP. This may be attributed to the fact that, although the organisms are obtaining its P requirement from insoluble source, all may not be equally efficient and may also have different ways to make it available. Most of the soluble P (K_2HPO_4) was present as available P and the available P in case of insoluble phosphate sources showed gradual increase with incubation time. It shows that the organisms were continuously solubilizing the insoluble phosphates and a part of it was utilized by them for their own growth and metabolism, and the remaining was left in the medium. There was in fact only a marginal increase in available P in cell free supernatants of cultures supplied with insoluble phosphates. The higher concentrations of insoluble phosphate in medium did not seem to have much effect on the amount of available P and there seems to be an optimum concentration after which the amount of available P from insoluble phosphates remains more or less constant. Roychoudhury and Kaushik [4] also observed varying quantities of available P in the culture filtrates of BGA in presence of MRP as P source, but the differences observed were low and due to different organisms used. The results also showed that not all insoluble phosphate could be brought into soluble form and a major portion still remained insoluble. This may be because certain portions of insoluble phosphates remain bound and are not solubilized by microorganisms. The 'P' solubilizing activity of microorganisms is also sometimes regulated by the presence of soluble phosphates [14].

The nitrogen fixation activity shown by cyanobacterial strains in normal BG-11 medium with soluble P source was also seen to be maintained in presence of insoluble phosphates also (Table 2). Normally, the nitrogenase activity reaches its peak and then decreases quickly as the culture becomes old. But in our study where insoluble 'P' source is utilized, the nitrogenase enzyme activity gradually increased and formed a plateau to maintain enhanced activity for relatively longer period of incubation. This may be due to readily available P in form of K_2HPO_4, the organisms were having adequate amount of available 'P' in the medium from the beginning, and it was readily utilized to show normal nitrogenase activity. But, in the case of insoluble phosphate source (MRP and TCP), the 'P' was slowly made available to the organisms and it took a longer time to show gradually higher activity. It may also be true that due to P stress, the organisms were not able to maintain their normal growth and metabolic activity in presence of MRP and TCP and might have resulted in higher heterocysts and nitrogen fixation. Table 1 does not show a

Table 2 Time course effect of different phosphate sources on acetylene reduction (AR) activity* and available phosphorus in cell free supernatant of cyanobacteria

Treatments (T)		Control (-P)	K_2HPO_4	MRP (mg P l^{-1})			TCP (mg P l^{-1})			Mean (SXD)	Mean (S)
Strains (S)	Days (D)			10	20	30	10	20	30		
Westiellopsis prolifica	7	0.30 (0.28)	0.63 (3.63)	0.32 (0.12)	0.31 (0.20)	0.26 (0.32)	0.78 (0.12)	0.49 (0.26)	0.55 (0.38)	0.46 (0.66)	2.82 (0.45)
	14	0.68 (0.29)	5.58 (2.85)	2.52 (0.11)	1.48 (0.16)	0.85 (0.27)	4.93 (0.12)	6.92 (0.22)	4.24 (0.32)	3.40 (0.55)	
	21	0.29 (0.36)	1.04 (2.16)	3.16 (0.11)	3.19 (0.15)	1.22 (0.27)	6.07 (0.10)	9.42 (0.18)	6.97 (0.27)	3.92 (0.45)	
	28	0.24 (0.33)	1.03 (1.43)	7.62 (0.05)	5.83 (0.13)	5.64 (0.22)	4.35 (0.07)	4.46 (0.13)	7.94 (0.25)	4.64 (0.33)	
	35	0.22 (0.28)	1.00 (0.98)	2.24 (0.04)	1.75 (0.11)	1.90 (0.22)	1.93 (0.06)	2.07 (0.11)	2.52 (0.23)	1.70 (0.25)	
Mean (SXT)		0.35 (0.31)	1.85 (2.21)	3.17 (0.09)	2.51 (0.15)	1.97 (0.26)	3.61 (0.09)	4.67 (0.18)	4.44 (0.29)		
Anabaena variabilis	7	0.35 (0.31)	0.64 (3.60)	0.24 (0.10)	0.34 (0.24)	0.31 (0.35)	0.86 (0.11)	0.75 (0.24)	0.80 (0.37)	0.54 (0.67)	3.03 (0.46)
	14	0.85 (0.24)	3.07 (3.03)	1.35 (0.10)	1.50 (0.23)	1.49 (0.28)	2.67 (0.10)	2.92 (0.21)	2.37 (0.34)	2.03 (0.57)	
	21	0.25 (0.24)	5.90 (2.26)	2.21 (0.09)	2.22 (0.20)	2.23 (0.27)	4.19 (0.07)	10.42 (0.18)	5.54 (0.34)	4.12 (0.46)	
	28	0.14 (0.25)	8.77 (1.41)	4.14 (0.05)	5.78 (0.18)	4.31 (0.25)	3.27 (0.06)	8.10 (0.18)	6.26 (0.30)	5.10 (0.34)	
	35	0.11 (0.18)	3.61 (1.22)	3.14 (0.04)	2.36 (0.15)	2.75 (0.26)	1.64 (0.05)	7.43 (0.17)	5.86 (0.28)	3.36 (0.30)	
Mean (SXT)		0.34 (0.24)	4.40 (2.30)	2.22 (0.08)	2.44 (0.20)	2.22 (0.28)	2.52 (0.08)	5.93 (0.20)	4.17 (0.33)		
TXD	7	0.33 (0.30)	0.64 (3.62)	0.28 (0.11)	0.33 (0.22)	0.28 (0.33)	0.82 (0.12)	0.62 (0.25)	0.68 (0.37)		
	14	0.77 (0.27)	4.33 (2.94)	1.94 (0.11)	1.49 (0.20)	1.17 (0.28)	3.80 (0.11)	4.93 (0.22)	3.31 (0.33)		
	21	0.27 (0.30)	3.47 (2.21)	2.69 (0.10)	2.70 (0.18)	1.72 (0.27)	5.13 (0.09)	9.92 (0.18)	6.25 (0.31)		
	28	0.19 (0.29)	4.90 (1.42)	5.88 (0.05)	5.80 (0.15)	4.98 (0.24)	3.81 (0.06)	6.28 (0.16)	7.10 (0.28)		
	35	0.16 (0.23)	2.30 (1.10)	2.69 (0.04)	2.05 (0.13)	2.32 (0.24)	1.78 (0.06)	4.75 (0.14)	4.19 (0.26)		

	S	TXS	TXD	SXD	TXSXD
SEM±	0.0031 (0.0052)	0.0087 (0.0147)	0.0137 (0.0233)	0.0069 (0.0117)	0.0194 (0.0330)
CD ($P = 0.05$)	0.0086 (0.0144)	0.0241 (0.0407)	0.0380 (0.0646)	0.0191 (0.0324)	0.0538 (0.0915)

* Values are μ mole C_2H_4 $^{-1}$mg chlorophylll^{-1} h^{-1}

Figures in parenthesis represent available phosphorus and values are µg available P ml^{-1}

Table 3 Time course effect of different phosphate sources on heterocyst frequency (%)* in cyanobacteria

Treatments		Control (-P)	K_2HPO_4	MRP (mg P l^{-1})			TCP (mg P l^{-1})		
Strains	Days			10	20	30	10	20	30
Westiellopsis prolifica	7	3.0	6.3	3.2	3.1	2.6	7.8	4.9	5.5
	14	6.8	11.2	5.0	3.0	1.7	9.9	13.8	8.5
	21	2.9	2.1	6.3	6.4	2.4	12.1	18.8	13.9
	28	2.4	2.1	15.2	11.7	11.3	8.7	8.9	15.9
	35	2.2	2.0	4.5	3.5	3.8	3.9	4.1	5.0
Anabaena variabilis	7	3.5	6.4	2.4	3.4	3.1	8.6	7.5	8.0
	14	8.5	7.7	3.4	3.8	3.7	6.7	7.3	5.9
	21	2.5	14.8	5.5	5.6	5.6	10.5	26.1	13.9
	28	1.4	21.9	10.4	14.5	10.8	8.2	20.3	15.7
	35	1.1	9.0	7.9	5.9	6.9	4.1	18.6	14.7

*Ratio of heterocysts to total number of cells in atleast 10 randomly selected filaments

reduced growth where MRP and TCP were used as sole 'P' source as much of the insoluble P was made available P. This may be another reason for maintenance of nitrogen fixation under these conditions [15, 16]. Also the relation between the mean available P in the medium was compared for the mean AR activity irrespective of the days of incubation (Table 2) and it was observed that *A. variabilis* and *W. prolifica* showed best mean AR activity at TCP (20 mg P l^{-1}) more than the positive control (K_2HPO_4). It was also concluded that among the two cyanobacteria *A. variabilis* was best in terms of P-solubilization and nitrogen fixation and TCP (20 mg P l^{-1}) was the best source of insoluble P rather than MRP or K_2HPO_4.

Cyanobacteria are considered to be an efficient sink for P, thus continuously causing P-transformations. It has been shown that like other microorganisms, P is necessary for the growth and nitrogen fixation by these organisms. Therefore, it seems to be highly significant correlation between P-solubilization/availability and nitrogen fixation activity during incubation. The nitrogenase activity is however, drastically affected in P-deficient cultures. The results of current study are in agreement with the above reports.

Anabaena variabilis and *Westiellopsis prolifica* were capable of nitrogen fixation and showed luxuriant growth and metabolic activity in medium containing MRP or TCP. These can, therefore, be exploited for the efficient utilization of low cost, low grade rock phosphate fertilizers where phosphate rock can be applied directly. This would be especially beneficial in developing countries with limited P resources. Besides, dissolution of inorganic P, these organisms can also mobilize organic P fraction of the soil due to their ability to synthesize phosphatases [7] and making it available to plants.

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The heterocystous cyanobacteria *A. variabilis* seems to be an ideal choice for the preparation of microbial inoculant because of its ability to grow well and fix atmospheric nitrogen in the absence of combined nitrogen and soluble phosphates. The study clearly demonstrated that preference of source of insoluble phosphate to solubilize P is a strain dependent attribute and the cyanobacteria could sustain nitrogenase activity for a longer duration before reaching a plateau in presence of insoluble phosphate as compared to soluble phosphate source.

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