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Short Communication

Comparative efficacy of qualitative and quantitative methods for rock phosphate solubilization with phosphate solubilizing rhizobacteria

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Abstract

Some plant growth promoting rhizobacteria (PGPR) have the ability to solubilize the insoluble inorganic forms of phosphorus (P) through the production of specific metabolites such as organic acids. These phosphate solubilizing bacteria (PSB) are routinely screened by qualitative method (NBRIP medium agar plate assay) by observing the size of a halo/ clear zone on plate around the bacterial colony due to production of organic acids into surrounding medium. In this study, this qualitative method was compared with quantitative (colorimetery) method of measuring the P solubilization by PSB to evaluate the validity/accuracy of the former method. Results of quantitative and qualitative methods indicated that qualitative method did not reflect the real potential of PSB to solubilize insoluble P as no relationship was observed when values regarding P solubilization by PSB determined by qualitative method were regressed against respective values recorded for quantitative method. Interestingly, same strains which showed very poor performance in qualitative method were proven good P solubilizers in quantitative method and vice versa. It could be concluded that only quantitative method should be considered reliable when studying P solubilization by phosphate solubilizing microbe (PSM).

Key words: Phosphate solubilizing rhizobacteria, Qualitative, Quantitative, Assay

It is well documented that major fraction of soil phosphorus are usually present in the forms which are unavailable to plant (Zaidi, *et al.,* 2009). Some microorganisms carry substantial potential to solubilize this unavailable fraction of soil P and those are generally termed as phosphorus solubilizing microorganism (PSM) (Hameeda *et al.,* 2008; Henri *et al.,* 2008). The PSM are particularly of great use to circumvent the problem of low P availability in calcareous soils. At present, several bacteria including *bacillus, rhizobium* and *pseudomonads* are the most studied phosphate solubilizers (Rodriguez and Fraga, 1999; Wani *et al.,* 2007). Phosphorus solubilizing microorganism produce organic acids such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate, etc. (Gyaneshwar *et al*., 1999; Puente *et al*., 2004; Song *et al.,* 2008; Trivedi and Sa, 2008) which help in solubilization of insoluble P in the medium. Mostly this ability of various PSM is determined by observing halo/clear zone on plate due to the production of organic acids into the surrounding medium (Nautiyal, 1999; Katznelson, *et al.,* 1962). The comparative potential of various PSM is routinely determined by screening through this qualitative assay of measuring the size of halo zone arround the colony on the plate (Nautiyal, 1999; Pikovskaya, 1948). Many researchers are still using this qualitative method (plate assay) to assess the P solubilizing ability of PSB by observing clear halo arround colony of PSB. However, the reliability of this qualitative method (halo based technique) has yet not been well estabilished and need to be determined as many isolates which did not produce any visible halo/zone on agar plates were found capable of solubilizing various types of insoluble inorganic phosphates in liquid medium (Louw and Webley, 1959; Gupta, *et al.,* 1994). Thus it is very likely that the information collected through qualitative method may be misleading untill and unless it is correlated with quantitative method. Quantitative method reflect the actual picture of P solubilization by PSM. In quantitative method the amount of soluble P released from insoluble substrate as a result of microbial activity is measured colorimetery.

The objectives of the present study were to estimate the comparative reliability of qualitative method as compared with that of quantitative method by using bacterial solubilization of rock phosphate. The results of this study will be helpful to screen PSM for future research.

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A study was conducted to evaluate the comparative reliability of qualitative method with that of quantitative method by using rock phosphate as substrate and measuring the phosphate solubilizing activity of several rhizobacteria under axenic conditions.

Isolation of rhizobacteria

For isolation of rhizobacteria containing phosphate solubilizing activity, rhizosphere soils were collected from wheat and maize plants. Rhizobacteria were isolated by dilution plate technique (Wollum II, 1982) using general purpose medium. The collected rhizobacterial isolates were purified by further streaking on fresh plates of national botanical research institute's phosphate (NBRIP) medium (Nautiyal, 1999) using rock phosphate as sole phosphorus source. While NABRIP medium contained per liter: glucose, 10g; $Ca_3(PO_4)_2$, 5g; $MgCl_2.6H_2O$, 5g; $MgSO_4.7H_2O$, 0.25g; KCl, 0.2g; $(NH_4)_2SO_4$, 0.1g. The flasks were incubated at 28±1ºC for 5 days in the orbital shaking incubator at 100 rpm. An optical density of 0.5 at λ 550 nm was achieved by dilution to maintain uniform cell density (10^7 - 10^8 cfu mL^1).

Solubilization of inorganic phosphate

Phosphorus solubilizing activity was determined qualitatively and quantitatively according to the method described by Nautiyal (1999).

Qualitative phosphate solubilization

Bacterial strains were evaluated for their ability to solubilize inorganic phosphate. Agar medium containing calcium phosphate as the inorganic form of phosphate was utilized in this assay. Bacterial strains were tested qualitatively by plate assay using NABRIP medium. Bacterial strains were cultured in NBRIP broth for 48 h and inoculated on NBRIP agar plates separately. A loop full of each culture was placed on the plates; one per plate using sterile toothpicks and the plates were incubated at 30°C for 7 days. Appearance of halo zone around the colonies after 5 days incubation indicated phosphate solubilization. The experiment was performed thrice with three replicates for each bacterial strain. Halo size produced by the respective strain was measured and categorized in to low, medium and high on the basis of zone diameter. The strain making a zone of diameter <1 cm was considered as low P solubilizer, medium with a diameter of halo 1-2 cm and high having a diameter of halo more than 2 cm.

Quantitative phosphate solubilization

Quantitative estimation of phosphate solubilization in broth was carried out using Erlenmeyer flasks (100 mL) containing 20 mL of medium inoculated in triplicate with the bacterial strains. Sterile uninoculated medium was used as negative control. The flasks were incubated for 2 days at 30°C on a shaking incubator at 100 rpm. The cultures were harvested by centrifugation at 10000 rpm for 10 min using centrifugation machine (Sigma 216 KC, Germany). Phosphorus in culture supernatant was estimated by using method as described (Ryan, *et al.,* 2001). The absorption of the supernatant was measured at 410 nm by using (Nikolet evolution 300) spectrophotometer. The data are means of three experiments with three repeats. P solubilizations by strains were categorized into low, medium and high by assigning value 0, 1 and 2 by using Score Indexing technique as described by Gill *et al.* (2004).

Comparative solubilization of rock phosphate by qualitative method through observing halo zone on agar plate and in quantitative method, (NBRIP broth) of phosphate solubilization was examined colorimetery for the thirty five bacterial strains. All the strains were showing different size of halo zone in qualitative method (Table 2) and different P solubilization in quantitative method (Table 1). The results revealed that both the methods did not follow the same trend for the tested bacterial strains, for example, the strain which showed little or no halo around the colony in qualitative assay was found as the most efficient strain in quantitative assay. Phosphate solubilization potential (halo/clearing zone) of the strains RM19 (0.7 cm) & RW27 (0.8 cm) was low in qualitative method, but these strains showed quite high phosphate solubilization activity as measured by quantitative method (Table 3). Similarly the strains RM5 (0.0 cm), RM28 (0.3 cm), RM12 (0.7 cm), RM13 (0.7 cm), RW11 (0.6 cm), RW31 (0.8 cm), RW23 (0.6 cm) and RW8 (0.7 cm) exhibited least clearing zone around colonies in qualitative method, while these strains showed medium phosphate solubilization potential in quantitative method. Contrarily, strains producing halo zone in agar plates showed poor performance with respect to solubilization in colorimetric method. However, very interestingly, strains RM35 (2.6 cm), RM21 (1.8 cm) and RM25 (3.5 cm) produced big clearing zone/halo zone around colonies in qualitative method, also proved very promising in quantitative method (Table 3). When the values of qualitative method were regressed against values recorded in case of quantitative method, no relationship was found (Figure 1). All these findings revealed that one should not rely only on qualitative method while isolating and screening the PSM. It is wise to supplement qualitative method with quantitative measurement of P solubilizing for getting more reliable inferences. Similar results have been reported by Gupta, *et al.* (1994); Nautiyal (1999) and Nautiyal, *et al.* (2000). It has also been reported that many isolates which did not show any clear zone in qualitative method i.e. NBRIP medium- agar plate assay) solubilized insoluble

Table 1. Quantitative P-solubilization of rock phosphate by phosphate solubilizers

Sr. No.	Strains	Quantitative solubilization (µg mL^{-1})	Index Scoring
1	RM5	0.00	0
2	RW6	180.10	0
3	RM22	113.30	0
4	RM1	185.32	0
5	RM 3	240.50	0
6	RM 2	571.45	1
7	RW9	548.36	1
8	RM4	532.73	1
9	RW11	582.01	1
10	RM 13	584.50	1
11	RM16	603.95	1
12	RW29	583.77	1
13	RW 8	398.26	1
14	RM 7	528.18	1
15	RM 17	637.70	1
16	RW 20	503.64	1
17	RM10	461.20	1
18	RW34	514.62	1
19	RW 32	546.92	1
20	RM12	619.48	1
21	RM 14	509.13	1
22	RW33	528.59	1
23	RW 31	549.71	1
24	RM 28	572.28	1
25	RM 24	501.37	1
26	RM 30	396.92	1
27	RW15	497.64	1
28	RW23	372.90	1
29	RM 26	516.69	1
30	RM18	661.05	1
31	RM 19	686.00	2
32	RW 27	678.59	2
33	RM 35	673.95	2
34	RM 21	698.50	2
35	RW25	753.00	2

inorganic phosphates in quantitative method (Louw and Webley, 1959; Leyval and Barthelin, 1989; Nautiyal, 1999). Thus, the existing plate assay fails where the halo is inconspicuous or absent. This may be because of the varying diffusion rates of different organic acids secreted by an organism (Johnston, 1952). Contrary to qualitative method (indirect measurement) of phosphate solubilization by plate assay, the quantitative method (direct measurement) of phosphate solubilization in broth assay resulted in reliable results (Nautiyal, 1999).

Table 2. Qualitative P-solubilization of rock phosphate by phosphate solubilizers

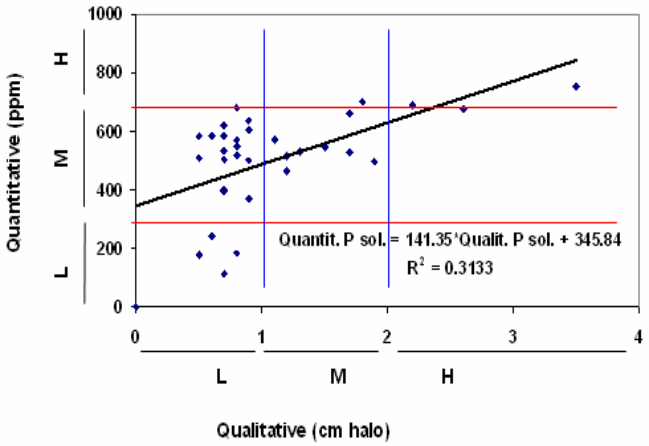
Sr. No.	Strains	Qualitative solubilization (cm)	Categorized
1	RM5	0	0
2	RW6	0.5	0
3	RM22	0.7	0
4	RM1	0.8	0
5	RM 3	0.6	0
6	RM 2	0.8	0
7	RW9	1.5	1
8	RM4	0.7	0
9	RW11	0.6	0
10	RM 13	0.7	0
11	RM16	0.9	0
12	RW29	0.5	0
13	RW 8	0.7	0
14	RM 7	1.7	1
15	RM 17	0.9	0
16	RW 20	0.7	0
17	RM10	1.2	1
18	RW34	0.8	0
19	RW 32	1.5	1
20	RM12	0.7	0
21	RM 14	0.5	0
22	RW33	1.3	1
23	RW 31	0.8	0
24	RM 28	0.3	1
25	RM 24	0.9	0
26	RM 30	0.7	0
27	RW15	1.9	1
28	RW23	0.6	0
29	RM 26	1.2	1
30	RM18	1.7	1
31	RM 19	0.7	2
32	RW 27	0.8	0
33	RM 35	2.6	2
34	RM 21	1.8	1
35	RW25	3.5	2

Table 3. Comparative efficacy of quantitative Vs qualitative method of rock phosphate solubilization

Phosphate Solubilization					
High		Medium		Low	
Qualitative Methods	**Quantitative Method**	**Qualitative Methods**	**Quantitative Method**	**Qualitative Methods**	**Quantitative Method**
RM21, RM25, RM35	RM21, RM25, RM35	RM2, RM7, RM18, RW9, RW15, W32, RW33, W34	RM2, RM7, RM18 RW9, RW15, RW32, RW33, RW34	RM1, RM3, RM22 RW6	RM1, RM3, RM22, RW6
	RM19, RW27			RM19, RW27	
			RM4, RM10, RM13, RM14, RM16, RM17, RM24, RM26, RM28, RM30, RW5, RW8, RW11, RM12, RW20,RW23, RW29, RW31	RM4, RM10, RM13, RM14, RM16, RM17, RM24, RM26, RM28, RM30, RW5, RW8, RW11, RM12, RW20, RW23, RW29, RW31	

Conclusion

Therefore, it is hereby suggested that microbes from soil should be screened by quantitative method (NBRIP broth) rather than qualitative method (NBRIP- Agar plate assay) for the identification of the most efficient phosphate solubilizers.



* L, low; M, medium; H, high

Figure 1. Correlation between Quantitative and Qualitative methods

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