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Solubilization of Rock Phosphates by *Rhizobium* and *Bradyrhizobium*

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ABSTRACT. The ability of *Rhizobium* and *Bradyrhizobium* strains to solubilize phosphate from hydroxyapatite was determined in a medium containing NH_4Cl or KNO_3. The presence of NH_4^+ in the medium resulted in higher solubilization of phosphate as compared to the presence of KNO_3, with the exception of *R. leguminosarium* bv. viceae strain TAL 1236 and 1402 which solubilized comparable amounts of phosphate in a medium containing either KNO_3 or NH_4Cl. These results suggest that the strains employ two different mechanisms for phosphate solubilization, one depending on the presence of NH_4^+, the other not requiring its presence. Temperature and aeration (O_2 demand) optima were 30 °C and 4.2 Hz (shaking frequency), respectively. In nonsterile soil the tested strain (*R. meliloti* TAL 1236) was very effective in solubilizing rock phosphate.

The use of rock phosphate as P-fertilizer, its solubilization through microorganisms and the increase of available P in soil solution have become the center of interest (Asea *et al.* 1988; Babenko *et al.* 1984; Halvorson *et al.* 1990). Several authors attribute the solubilization of inorganic insoluble phosphate by microorganisms to the production of organic acids and chelating oxo acids from suggars (Beever and Burns 1980; Kucey 1983; Leyval and Berthelin 1989).

The aim of this study was to investigate the capability of strains of *Rhizobium* and *Bradyrhizobium* to solubilize phosphate from hydroxyapatite and to determine their demand on culture conditions (temperature and aeration) and nutrition.

MATERIALS AND METHODS

Organisms. The strains of *Rhizobium* (TAL) and *Bradyrhizobium* (CB) were procured from *Rhizobium* germ plasm Resource, *University of Hawaii*, NifTAL project and MIRCEN, Paia, Hawaii (USA) and *Cunningham Laboratory*, St. Lucia, Queensland (Australia).

Media and culture. The medium contained (mg/L) $MgSO_4 \cdot 7H_2O$ 500, $FeCl_3$ 2, $MnSO_4$ 2 and NH_4Cl 1000. The pH of the medium was adjusted to 7.0 before autoclaving. Autoclaved mannitol was added to the medium to a final concentration of 1 %. Membrane-sterilized solutions of biotin, calcium pantothenate and thiamin hydrochloride were added to the medium aseptically, each to a final concentration of 1 mg/L. Insoluble hydroxyapatite was sterilized separately and then mixed with the autoclaved medium to a final concentration of 0.2 % (*W/V*). To study the effect of nitrogen source on the solubilization of hydroxyapatite, NH_4Cl was replaced with KNO_3 (1 g/L).

Flasks containing 25 mL of medium were inoculated with 1 mL stationary-phase cells, previously grown in a medium of similar composition but containing 0.1 % Na_2HPO_4 and incubated on an orbital shaker at 1.8 Hz and 28 °C for 3 d. Growth was measured turbidimetrically at 520 nm.

Estimation of phosphorus. Cultures were centrifuged (5 000 *g*, 15 min) to remove biomass and unsolubilized matter; soluble phosphate was determined in an aliquot of the supernatant according to Chen *et al.* (1956).

Rock phosphate solubilization in soil. Fifty grams of unsterilized clay soil (pH 7.4, total nitrogen 0.08 %, total soluble salts 0.23 %, organic matter 2 %, phosphorus 6 ppm) was mixed with rock phosphate at 0.2 % (*W/W*). The soil was inoculated with 2 mL of *Rhizobium meliloti* strain TAL 1372 suspension. Two controls, one soil with rock phosphate but without *Rhizobium*, other with *Rhizobium* but without rock phosphate, were used. After 20 d of inoculation the soluble phosphorus was extracted with $NaHCO_3$ (0.5 mol/L) and estimated.

Statistical analysis. Statistical analysis was done by means of one-way analysis of variance (PC-state computer program). Means were separated by using Duncan's multiple range test.

RESULTS AND DISCUSSION

Effect of nitrogen source

Most of the tested strains solubilized phosphate from hydroxyapatite with a decrease in medium pH in the presence of NH_4Cl as a sole source of nitrogen (Table I). Among the strains of *Rhizobium*, all the members dissolved very high levels of phosphate, the value ranging from 135 to 225 mg P/L of culture with the exception of *Rhizobium* sp. CB 3060 (*Leucaena leucocephala*) and *R. meliloti* TAL 380. On the other hand, all the strains of *Bradyrhizobium*, except CB 1024 (*Lablab purpureus*) solubilized low levels of phosphate, ranging from 0 to 13 mg P/L culture. The strain CB 627 (*Desmodium intortum*) did not appreciably reduce the medium pH and was unable to solubilize any detectable phosphate. Since the strain CB 627 grew appreciably in the absence of any detectable amount of phosphate solubilization, it is possible that the strain solubilized only low levels of phosphate which was consumed for growth. Using KNO_3 as a sole source of nitrogen caused a decreased solubilization of phosphate from hydroxyapatite by *Rhizobium* and *Bradyrhizobium* strains, except for *R. leguminosarum* strains TAL 1236 and 1402. These strains solubilized very high levels of phosphate (235 and 205 mg P/L) in a medium containing NO_3^- rather than NH_4Cl (192 and 186 mg P/L). These differences could be attributed to the employment of different mechanisms for the generation of acidity in the cultures. The presence of NH_4^+ in the growth medium of *Penicillium cyclopium* was reported to result in the development of inorganic acid following an operation of NH_4^+/H^+ exchange mechanism (Roos and Luckner 1984). Cabala-Rosand and Wild (1982) attributed higher phosphorus concentrations in plants amended with rock phosphate and NH_4^+ to acidification of the rhizosphere.

Table I. Effect of nitrogen source on phosphate solubilization from hydroxyapatite by *Rhizobium* and *Bradyrhizobium*[a]

Strain	NH_4Cl			KNO_3		
	absorbance	final pH	P mg/L	absorbance	final pH	P mg/L
R. leguminosarum bv. *viceae* (Pea)						
TAL 1236	1.13	3.1	192	0.56	3.9	235
TAL 1402	0.92	4.3	186	0.42	4.2	205
R. meliloti (Alfalfa)						
TAL 380	0.52	5.1	50	1.42	5.6	10
TAL 1372	1.02	4.1	225	1.65	4.1	0
TAL 1373	0.89	4.8	136	1.50	4.5	33
Rhizobium sp. (Leucaena)						
CB 3060	0.73	5.2	58	0.42	5.8	12
Rhizobium sp. (Chikpea)						
TAL 620	0.43	4.2	180	0.22	5.3	118
TAL 480	0.35	4.6	155	0.19	5.6	105
Bradyrhizobium sp.						
CB 2272 (Lupin)	0.36	5.9	11	0.11	6.4	7
CB 1024 (Lablab)	0.54	4.2	120	0.25	5.0	15
CB 627 (Desmodium)	0.83	6.4	0	0.37	6.3	8
CB 1015 (Cowpea)	0.32	5.7	13	0.18	6.1	6
CB 756 (Siratro)	0.22	5.9	8	0.13	6.3	7

[a]Each value represents the mean of three replicates.

It appears that among the different mechanisms employed by strains of *Rhizobium* and *Bradyrhizobium*, one relied on the presence of NH_4^+ in the medium. The operation of this mechanism resulted in the production of inorganic acid (reduction in pH culture) which causes high solubilization of phosphate. However, a second mechanism did not require the presence of NH_4^+ and probably involved the excretion of organic acid metabolites as also shown by Asea *et al.* (1988) and Thomas (1985).

The results of the present study revealed that *R. meliloti* strain TAL 1372 relied exclusively on the first mechanism. However, *Rhizobium* strains TAL 620 and 480 (Cicer arietinum) employed both mechanisms for phosphate solubilization.

Effect of temperature

Liquid cultures of *R. meliloti* TAL 1372 were grown at temperatures ranging from 15 to 40 °C, the optimum temperature being 30 °C. Although the pH of the medium decreased from about 7 to 3.9 during growth of *R. meliloti* at 20 °C, phosphate solubilization occurred to a much lower extent than at the optimum temperature which indicates that acid production is not the only reason for phosphate release into the medium.

Effect of aeration (O_2 demand)

For the estimation of the O_2 demand of *R. meliloti* strain TAL 1372 for phosphate solubilization the phosphate concentration was measured after 3 d at four different shaking speeds (0.83, 1.7, 2.5 and 4.2 Hz in Erlenmeyer flasks). The results indicate that phosphate solubilization was highest on shaking at medium speed (Table II). This could be attributed to the production and release of organic acids (pH decrease to about 3.4) which depends on the intensity of aeration of the shaken batch culture.

Table II. Effect of aeration (O_2 demand) on phosphate solubilization by *R. meliloti* strain TAL 1372[a]

Shaking frequency Hz	P, mg/L
0.83	53
1.7	186
2.5	230
4.2	345

[a]Each value represents the mean of three replicates. Values are significantly different at the 5 % level in Duncan's multiple range test.

Table III. Solubilization of phosphate from hydroxyapatite in nonsterile soil inoculated with *R. meliloti* strain TAL 1372[a]

Addition to soil	P, ppm
Hydroxyapatite	12
Rhizobium	25
Hydroxyapatite + *Rhizobium*	113

[a]Each value represents the mean of five replicates. Values are significantly different at the 5 % level in Duncan's multiple range test.

Phosphate-solubilizing rhizobia in soil

Since the conditions in soil are much more complex than those in the laboratory, after having obtained basic information about *Rhizobium* and *Bradyrhizobium* strains their ability for rock phosphate solubilization in nonsterile soil was investigated. Soluble phosphate increased distinctly compared to the controls (Table III). This indicates that even under nonsterile conditions *R. meliloti* strain TAL 1372 was very effective in solubilizing rock phosphate. Better knowledge of the solubilization mechanisms is indispensable for carefully directed application in agriculture.

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