Effect of Calcium on the Phosphorus Nutrition of *Rhizobium meliloti*[1]

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ABSTRACT

Effects of calcium at 300 and 1500 *μM* on P nutrition were assessed in eight strains of *Rhizobium meliloti* in defined liquid medium. Evaluations included: P storage from "luxury" external concentration (1000 *μM P);* utilization of stored P after transfer to unreplenished low-P medium (0.06 μ*M);* and growth at low concentrations of P buffered at 5, 0.5, and 0.06 *μM* with an iron oxide dialysis system. The strains stored P in luxury medium, but unlike other rhizobia, they needed high Ca to utilize the stored P. They either grew or died following transfer to low-P medium, depending on the Ca concentration and the Ca concentration at which they had grown previously. Ability to grow in media buffered at low P concentrations also contrasted with that of other rhizobia, in two respects: no strain of *R. meliloti* grew at 0.06 *μM P,* regardless of Ca concentration; and some strains needed high Ca to grow at 0.5 and 5 *μM* P. Two isolates from *Medicago rugosa* and *Melilotus indica* were less Ca-demanding than six isolates from *Medicago sativa.* Previous reports that *R. meliloti* has low calcium requirements may be correct only for the luxury P levels that are conventional in defined media. Our evidence for high Ca requirement at realistic P concentrations agrees with data from soil experiments.

Additional Index Words. **symbiotic N fixation, Fe oxide, goethite, P-buffering, uptake, utilization.**

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MINERAL NUTRITION STUDIES of plants and soil microbes in simplified artificial media become especially valuable when their results appear to contradict observations made in soils. Study of such discrepancies can uncover a previously unsuspected role for soil factors incorrectly reproduced in the artificial system. This paper reports such a case involving Ca and P nutrition of microbes important in nitrogen cycling, members of the genus *Rhizobium.*

There has long been evidence suggesting that in acid soils Ca deficiency can limit growth of *Rhizobium,* especially *R. meliloti* (9, 10,11). Yet all controlled studies of Ca nutrition of rhizobia in artificial media, even with *R. meliloti,* indicated requirements so low that Ca would seem unlikely ever to limit the organisms' growth in soil (2,14). The artificial media, as is conventional, contained millimolar concentrations of phosphate, hundreds of times higher than the concentrations actually encountered in soil solutions. Since Ca and P can interact in their transport into cells and organelles (5), we postulated a similar interaction in *R. meliloti,* such that high Ca becomes necessary when orthophosphate is lowered to concentrations relevant to the soil environment. This paper reports experiments testing the postulate, using a recently published procedure (1,6) for controlling phosphate concentrations in the micromolar range.

MATERIALS AND METHODS

Seven strains of *Rhizobium meliloti* were used: five isolates from *Medicago sativa* (Nitragin Co. strains 102F70 and 102F28 and United States Dep. of Agric. strains 1021a, 1029, and 1031), one isolate from *Medicago rugosa* (Nitragin 102H1), and one isolate from *Melilotus indica* (Nitragin 104B4). All the strains effectively nodulated *Medicago saliva* var. Moapa in aseptic agar tube culture. Cultures were maintained under refrigeration on yeast-agar slants similar in composition to our liquid luxury-P medium (below).

Arabinose-galactose (0.3% each) as an energy source was chosen because it did not interfere with the phosphomolybdate-blue determination of P. The liquid media also contained 1.1 g/L sodium glutamate, 0.1 mg/L biotin, and inorganic nutrients at the following micromolar concentrations: $MgS0_4$ 300; FeEDTA 50; $MnS0_4$ 2; $ZnS0_4$ 1; $CUS0_4$ 0.5; Na_2MoO_4 0.1; $CoC1_2$ 0.02. Calcium was supplied as chloride at two concentrations: 300 *μM* (low-Ca) and either 1500 μM or 3000 μM(high-Ca). The pH was adjusted to 5.5 with HCl before autoclaving. To maintain buffered concentrations of phosphate at low levels in solution, an "Fe oxide dialysis" system was used (6). Concentration of P in solution depended on the amount added with the iron oxide (powdered limonite from Ward's Natural Science Establishment, Rochester, NY). The oxide was phosphated in 150 g lots by shaking it for 6 d in 1.5 L of 10 mM $CaC1_2$ with the appropriate amount of KH_2PO_4, followed by filtration and air-drying. Each culture received a slurry of 3 g oxide and 4 mL water contained in a section of dialysis tubing knotted at both ends, added to 37 mL of medium in a 125 mL Erlenmeyer, culture flask, autoclaved 30 min, and left 3 d at 25 to 27°C to equilibrate. The desorption isotherm was then determined by analysis of the liquid media. The luxury-P control contained KH_2P0_4-K_2HP0_4 at a P concentration of 1000 *μM.* The low-P treatment, with no P added, and the oxide cultures, received 250 μM K_2SO_4 to ensure an adequate supply of K.

All liquid cultures were incubated at 25°C on an orbital shaker.

Three sets of experiments were done:

1. Phosphorus storage in cells was measured at both a luxury level of P (1000 μM) and a high level representing that found in solution in a fertile soil (5 μM). The 5 μM level was maintained using the oxide dialysis system. At each level of P, two replicates each of two levels of Ca (300 and 1500 *μM)* were inoculated and grown to 10^7 to 10^8 cells per mL, then centrifuged at relative centrifugal force 10 000Xg for 20 min, resus-

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pended in 10 mM $CaCl_2$ and recentrifuged. The pellet was dried overnight at 60°C, then analyzed after Kjeldahl digestion by a phosphomolybdate-blue procedure (13).

2. Growth was measured following transfer from luxury P (1000 μM) to unbuffered low-P (0.06 *μM)* medium. High-P cells grown at two levels of Ca were used to inoculate low-P medium containing the two levels of calcium in a factorial experimental design. The inocula were added to 37 mL media to give an initial density of about 10° cells per ml. Cell growth was followed by conventional drop counts on yeast arabinose-galactose plates. At each time (see Fig. 2 and 3), six replicate 41μL drops were counted from each triplicate culture. Analysis of variance was done on $\log_o$ transforms of the counts.
3. Growth was measured at buffered concentrations of phosphate likely to be encountered by bacteria in the soil, with the high level representing a P-fertile soil (5 *μM),* medium a P-deficient soil (0.5 *μM),* and low a P-depleted rhizosphere (0.06 *μM).* The inocula were from cultures grown in high-Ca, low-P medium to eliminate P storage effects, and diluted to provide 10^3 to 10^4cells per mL at the beginning of the experiment. Growth of strains 102F28, 102111, 104B4 and 1021a was monitored by drop counts at intervals for 7 d after inoculation into media at two levels of Ca and the three buffered levels of P, plus a 2000 *μM* P luxury control. The least significant difference (LSD, p=0.05) was calculated from the analysis of variance on $\log_{10}$ transforms of the counts. The culture medium was analyzed at 3 and 5 d for P to check the P-buffering performance of the oxide, and pH was checked near the end of each experiment.

RESULTS

Phosphorus storage was in the same range as in other *Rhizobium* species (1). At 1000 μ*M* P, the concentration of P stored varied from 1.3 to 1.9% of cell dry weight, depending on strain, but was unaffected by the level of calcium (Table 1). None of the strains grown with low Ca and 5 μM P achieved a high enough population for analysis of P. In the 5 μM P cultures, the rhizobia stored only enough P to support one to two generations following transfer.

Calcium level had a dramatic effect on the ability of *Rhizobium meliloti* strains to utilize their stored P upon transfer into low-P medium (Fig. 1). The amount of growth (or death) depended not only on the calcium level in the low-P media, but also on the calcium level at which the high-P inoculum had been grown. Stored P appeared sufficient to support three to six generations in high-Ca low-P medium, and growth following storage correlated fairly well with amount of P stored $R^2 = 0.85$ (Table 1). In the low-Ca low-P cultures, however, the number of viable cells decreased with time. The level of calcium at which the inocula were grown had a significant effect on subsequent growth to low P (Fig. 2 and 3). High calcium inoculum reached higher populations in high-Ca low-P medium, and remained viable longer in low-Ca low-P. Multiplication or survival varied with strain as well as calcium regime and amount of P stored.

Strain 102H1, isolated from *Medicago rugosa,* behaved similarly to the alfalfa strains in runout culture, while 104B4, isolated from *Melilotus indica,* would not grow at all in the low P media, regardless of cal

Table 1. Phosphorus accumulation by strains of *R. meliloti.*

Strain	mg P/g cell dry weight		Generations produced‡
	1000 *μM* P	5 *μM* P	
102F70(1)‡	18.6 a*	06.4 e	5.8 b
1021 a (2)	17.2 ab	05.6 ef	6.6 a
1031 (2)	15.2 b	04.0 f	4.2 c
102F28 (1)	12.7 c	04.3 f	3.0 d
1029 (2)	14.4 c	05.0 ef	4.4 c
102H1 (1)	15.0 b	06.2 e	4.2 c
104B4 (1)	15.2 c	04.0 f	no growth

* Means followed by different letters are significantly different at the 0.05 level.

† In high calcium, low P, 96 h after transfer from 1000 *μM* P to low (0.06 *μM*) P.

‡ Sources: (1) J. C. Burton, Nitragin Co., Milwaukee, WI. (2) H. H. Keyser, USDA, Beltsville, MD.

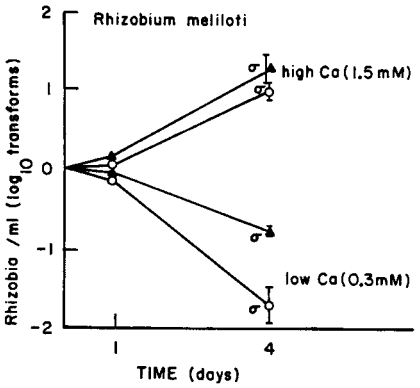

Fig. 1. Growth following transfer of cells from high-P into low-P medium at two levels of Ca. Inocula were grown at two Ca-levels: 300 *μM* (circles) and 1500 *μM* (triangles). The transforms are $\log_{10}$ (cell count/initial cell count). Standard deviations for treatments at day 4 are indicated by vertical lines. Data are averaged over strains.

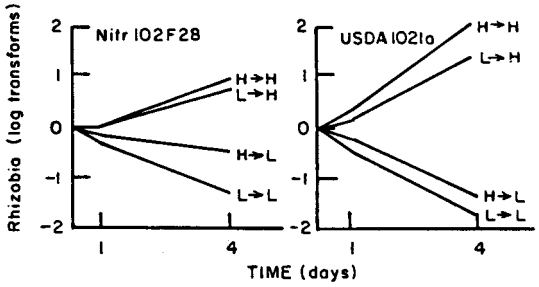

Fig. 2. Effects of Ca on growth of isolates from alfalfa after transfer from high-P into low-P medium. There were two Ca levels (H = 1500 *μM* and L = 300 *μM*), applied in four combinations of preinoculation and postinoculation treatment as shown. Log transforms as in Fig. 1.

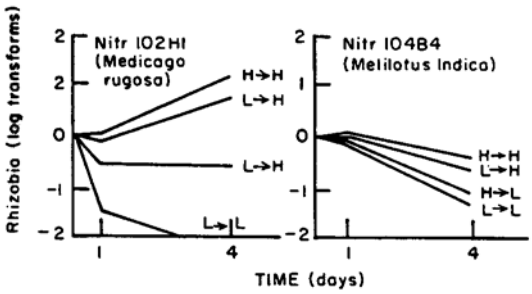

Fig. 3. "Runout" experiment on strains isolated from *Medicago rugosa* and *Melilotus indica.* Conditions and treatments as in Fig. 2.

cium level (Fig. 3). Phosphate storage for both was in the same range as in the other *R. meliloti* strains (Table 1).

The dialysis system buffered the phosphate concentration at low levels until populations in the cultures reached 10^6 to 10^7 cells per mL, at which point bacterial uptake exceeded the rate of P movement into solution (6). Accordingly, curves were plotted only as long as buffering capacity remained (through the first three days of growth).

Measurement of pH in culture solutions at the end of the growth period (7 d) indicated that all strains raised the pH of the media, with the magnitude of the shift corresponding to the population increase. The largest populations raised the pH to approximately 7.0, while pH remained at 5.5 where little or no growth occurred.

No *R. meliloti* strains grew at the lowest level of P (0.06 μM), regardless of calcium level. Differences in growth became significant in isolates from alfalfa at the medium P level (0.5 μM) after 72 h. (Fig. 4). Calcium concentration apparently affected the ability to utilize low solution P concentrations. Under the low calcium regime growth at 72h differed between the medium and high levels of P, but at high calcium there was essentially no difference in growth between these two P treatments (Fig. 4). Alfalfa isolates 102F70 and 102F28 produced an average of two generations with low Ca and medium P, and an average nine generations with high Ca and medium P after 72 h. At high P, and also in luxury P media (not shown) all the strains produced about the same population regardless of calcium level after 72 h.

The two isolates from *M. rugosa* and *M. indica* had somewhat different requirements than those from alfalfa. These two isolates grew almost as well at low Ca as at high Ca (Fig. 5). They also grew equally at the medium and high levels of P.

DISCUSSION

Uptake of P is generally considered an active process (3), and probably is dependent on an intact, normally formed structure of cell wall proteins. Calcium deficiency causes a loss of wall integrity, indicated by swollen, vacuolated cells and susceptibility to lysis and antibody absorption (15, 16). Magnesium is unable to overcome the wall deficiency. Addition of Ca to deficient cells does not restore normal morphology, indicating its role is played during cell wall formation. The structural role of Ca could be in the binding of otherwise free-COOH groups of the peptidoglycan layer (8), or by lending stability to the lipoprotein (14).

Bergersen (2) found that *R. meliloti* and *R. trifolii* had an increased lag phase and a shortened exponential phase in low Ca medium, while other species showed no difference with calcium treatments. All investigations into calcium requirements of *Rhizobium,* however, have been done at P levels of 1.0 mM or higher, and are representative of conditions likely to be found only in the laboratory. The synergistic effect of Ca and P that might take place in nature, where soil solutions are generally below 3 μM P (12) has not been previously considered. The data presented here show an interaction in solution culture at low levels of P.

Improvement in nodulation due to Ca has been attributed to several mechanisms (10), including increased rhizobial numbers in response to an increase

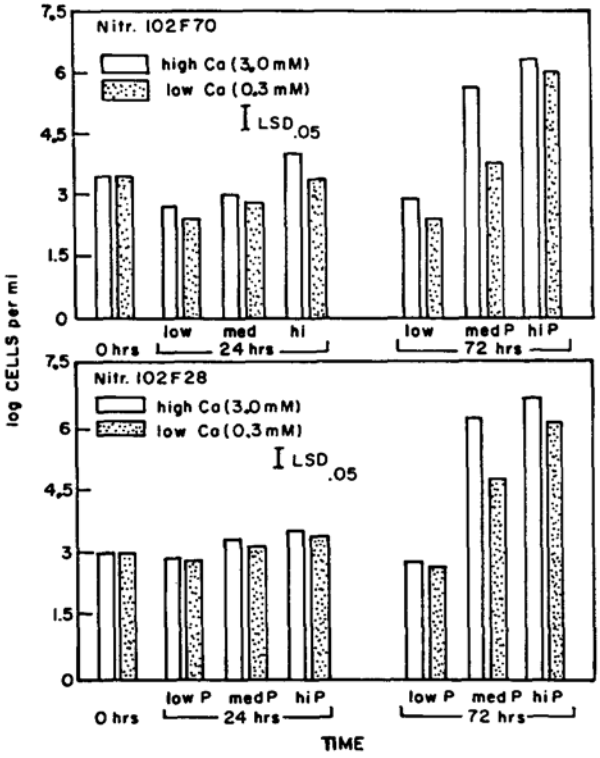

Fig. 4. Growth of two isolates from alfalfa at three buffered levels of P and two levels of Ca.

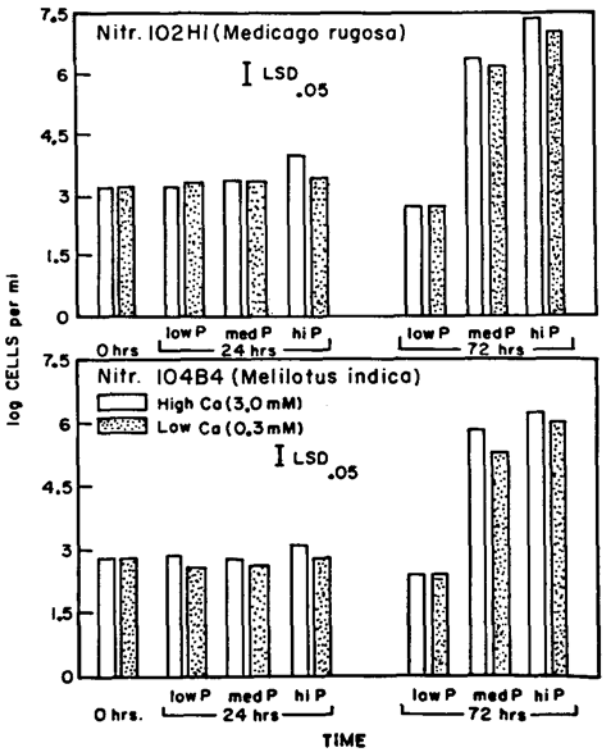

Fig. 5. Growth of isolates from *Medicago rugosa* and *Melilotus indica* at three buffered levels of P and two levels of Ca.

in calcium. In addition the direct effect of low Ca may have to do with the P nutrition of the rhizobia. This idea coincides with claims that P-fertilization has improved nodulation in greenhouse and field trials with several species (7, 17).

The greater sensitivity of *Rhizobium meliloti* to calcium deficiency, (2, 11), and subsequent P deficiency, suggests that the rhizobia co-evolved with hosts which are similarly sensitive (10). A loss in efficiency of extraction of Ca and P from the soil may have occurred as the symbiotic partners adapted to calcic conditions.

Phosphate requirements of the strains isolated from *Medicago rugosa* and *Melilotus indica* were less calcium dependent than those of isolates from alfalfa. Screening more alfalfa isolates might also uncover strains lacking susceptibility to calcium concentration. Further investigation is required to set limits on the Ca and P nutrition of *Rhizobium meliloti* as a whole, and to characterize cell morphology and behavior in a soil-plant system under calcium-induced phosphate stress.

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