PN-AAJ-955

Reprinted from the *Soil Science Society of America Journal*
Volume 45, no. 3, May-June 1981
677 South Segoe Rd., Madison, WI 53711 USA

NSF AER 77-07301 & AID/ta-c-1207 HC1365

Growth of *Rhizobium* Strains at Low Concentrations of Phosphate[1]

K. G. CASSMAN, D. N. MUNNS, AND D. P. BECK[2]

ABSTRACT

A technique was developed for assessing effects of P on growth, in defined liquid media at orthophosphate concentrations comparable to those in soil solutions. Phosphate was supplied in sorbed form on iron oxide (goethite) separated from the liquid medium by a dialysis membrane. Depending on the level of sorbed P, concentrations in solution were established in the range 0.05 to 30 μM. Analytical monitoring indicated that concentrations above 0.1 μM were buffered against depletion by cell growth until populations exceeded 10^8 cells per ml.

The system was used to compare P responses of seven strains of soybean and cowpea rhizobia grown from P-depleted inocula at initial densities of 10^4 cells per ml. Two P-efficient strains grew as rapidly at the lowest concentration (0.05 μM) as in a control medium at 2,000 μM. They would seem unlikely to be P-limited in the field. Other strains were less efficient. One strain could barely grow at P concentration below 1 μM; this strain could have difficulty colonizing P-deficient soils or normal rhizospheres.

Large differences in tolerance to low P among strains may be agronomically important. The technique could be simplified for routine screening of strains; and its operation at submicromolar concentrations could be improved for research on nutrition and requirements of P-efficient rhizobia and other organisms.

Additional Index Words: legumes, limonite, microbial nutrition, nodulation, nitrogen.

Cassman, K. G., D. N. Munns, and D. P. Beck. 1981. Growth of *Rhizobium* strains at low concentrations of phosphate. Soil Sci. Soc. Am. J. 45:520–523.

THERE HAS BEEN much research on phosphate nutrition of legumes but virtually none on phosphate nutrition of *Rhizobium* (1). The little relevant data imply that *Rhizobium* can grow at low concen-

[1] Contribution from Dep. of Land, Air, and Water Resources, Univ. of California, Davis CA 95616. Received 22 Aug. 1980. Approved 12 Dec. 1980.

[2] Postgraduate Research Scientist, Professor, and Graduate Research Assistant.

trations of dissolved orthophosphate, somewhere below 10 μM (5, 10). This ability would be expected; phosphate is typically between 1 and 10 μM in soil solutions (7) and it may fall below 0.1 μM in rhizospheres (4, 6). Rhizobia can produce effective nodulation on P-deficient plants. Yet sometimes P fertilization improves nodulation and nitrogen fixation (1, 3), perhaps because some strains of *Rhizobium* lack adaptation to normal low-P conditions. Loss of adaptation might, for instance, result from prolonged culture in conventional-culture media with P at concentrations 1,000 to 10,000 times higher than in soil solutions. When it is available at high concentration, strains do differ in ability to store P internally and use it for later multiplication under deficient conditions (2). But it seems likely that a strain capable of invading rhizospheres would also need efficient P uptake from very low ambient concentration. This property might also differ between strains.

In soil, release of P from the solid phase more or less buffers P in solution at low concentrations. Growth of organisms in P-deficient soil should then depend on the concentration maintained in the solution in contact with the organism. It will depend only incidentally on total P in the soil. Experiments in simple media will not be relevant if one simply adds different amounts of soluble P and allows them to run out. Concentrations must be maintained. This could be done in chemostat or flowing culture, but the large concentration ratio between organism and medium would cause difficulty, and screening of large numbers of strains would be awkward.

Accordingly, we developed an "iron oxide dialysis culture" system in which P is held in solution at constant concentrations by desorption and diffusion from phosphated iron oxide powder. For convenience of analysis and counting, the iron oxide is separated from the liquid medium by a dialysis membrane. The purpose of this paper is to describe the procedure and to present evidence for large differences between strains in their ability to grow at low external P concentrations.

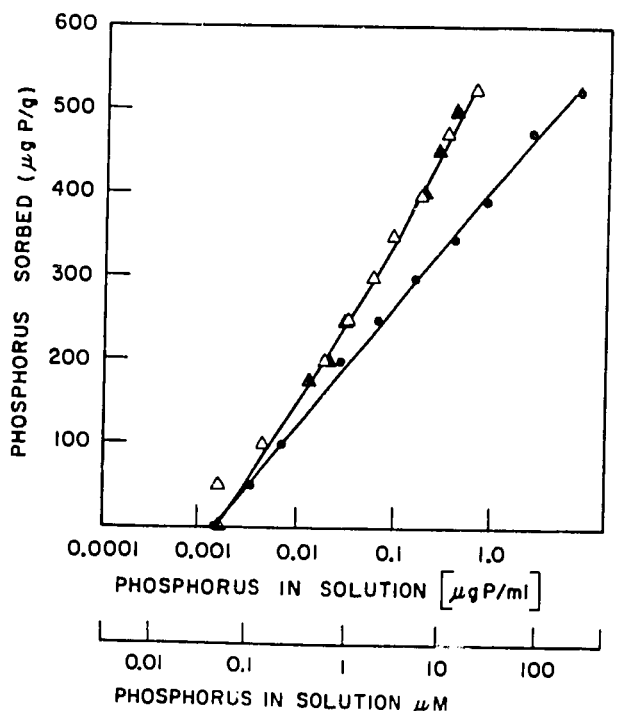

Fig. 1—Phosphate-sorption and -desorption isotherms for goethite (Ward's 'limonite"). Sorption isotherm (circles) by analysis of 0.01M $CaCl_2$ solution-phase at end of loading procedure. Desorption isotherm (triangles) by analysis of growth medium 3 days after addition of phosphated goethite with autoclaving. Black and open triangles indicate separately purchased and processed batches of go thite.

MATERIALS AND METHODS

Cultures of *Rhizobium japonicum* were obtained from the U.S. Department of Agriculture collection at Beltsville, Maryland. The cowpea *Rhizobium* strain CB756 (=TAL309) was obtained from CSIRO Cunningham Laboratory, St. Lucia, Queensland through the University of Hawaii NifTAL Project, Paia, Hawaii. Cultures were supplied and kept on yeast mannitol agar slants. Before use as inoculum, each strain was cultured in low-P ($<10^{-7}M$) galactose–arabinose glutamate medium to deplete stored P and adapt to the new carbon source.

The liquid experimental medium contained 3 g arabinose, 3 g galactose, and 1.1-g monosodium glutamate per liter of distilled water, with inorganic nutrients at the following micromolar concentrations: K_2SO_4 250; $CaCl_2$ 300; $MgSO_4$ 300; FeEDTA 50; $MnSO_4$ 2; $ZnSO_4$ 1; $CuSO_4$ 0.5; Na_2MoO_4 0.1; and $CoCl_2$ 0.02. The pH was adjusted to 5.5 with HCl. Phosphate was added either as KH_2PO_4 to concentration 2,000 μM (high-P control medium) or as a dialysis bag containing iron oxide appropriately preloaded with P.

The oxide was powdered limonite (Ward's Natural Science Establishment, Rochester, New York). According to x-ray diffraction analysis, it contained mainly goethite (αFeOOH), with minor quartz, mica, and kaolinite. The oxide was phosphated in 150-g lots by shaking 6 days in 1.5 liter of 10-mM $CaCl_2$ with the appropriate amount of KH_2PO_4 (Fig. 1), followed by filtration and air drying. The adsorption isotherm was determined at the end of the 6 days by analysis of the filtrate.

We used "Spectrapor" standard cellulose dialysis tubing, m.w. cutoff 12,000 to 14,000, diameter 16 mm, thickness 0.02 mm (Spectrum Medical Industries, Los Angeles, Calif.). To set up a culture, 3 g prepared oxide followed by 4 ml water were poured into a length of moistened tubing that had been knotted at the bottom. Air bubbles were removed while the slurry was mixed in the tube. The tube was then knotted at the top. One bag of oxide with 37-ml liquid medium was put into each 100-ml Erlenmeyer culture flask, autoclaved 30 min, and left 3 days at 25–26°C to equilibrate. The desorption isotherm (Fig. 1) was determined at the end of this time by analyses of the liquid media.

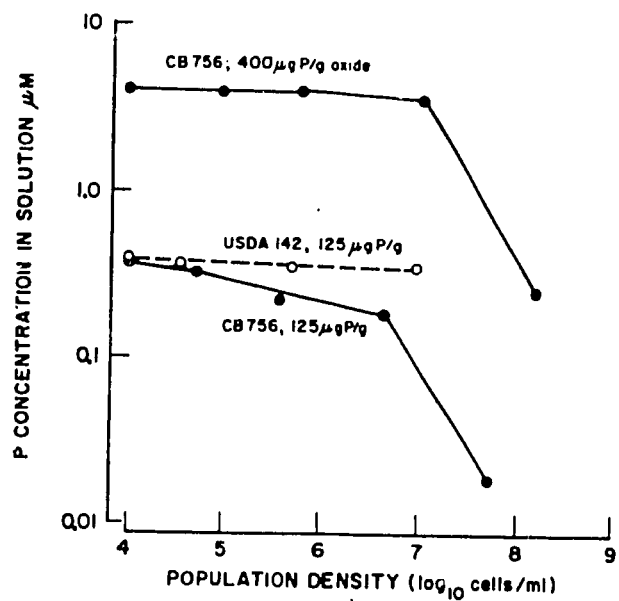

Fig. 2—Buffering of P concentration in relation to cell population density, showing failure of buffering at 10^7-cells per ml. Paired counts and P-analyses were made during growth in cultures inoculated at 10^4 cells per ml.

Phosphate concentrations were determined as phosphomolybdate reduced with ascorbic acid (8). Mannitol suppressed color development, but in galactose-arabinose medium we could detect no interference either by making analyses after standard additions or by varying sample size.

Experiments were inoculated from slightly turbid cultures (10^7 cells per ml) that had grown 3 days in arabinose-galactose medium without added P (2). Inoculum size was adjusted to provide 10^3 to 10^4 cells per ml of experimental medium at time zero. Viable cells were counted in 41-μl drops on plates of yeast mannitol agar (19). At each time, 6 replicate drops were counted from each triplicate culture. Analysis of variance was done on $\log_{10}$ transforms of the counts.

There were three sets of experiments:

1) Tests of buffering performance. Cultures with limonite preloaded at 125 and 400 μg P/g oxide were inoculated with strains CB 756 and USDA 142. Then on days 1, 2, 4, and 6, samples were counted and centrifuged and analyzed for P (Fig. 2). Similar results were obtained in a preliminary test with strain USDA 110.
2) Growth curves. Strains USDA 110, USDA 142, and CB 756 were counted daily after inoculation into media with oxide loaded at four levels of P. For USDA 142, a 2,000-μM control treatment was included. (Fig. 3).
3) Preliminary screening. Growth over 72 hours was determined by daily counting, for six strains of *R. japonicum* inoculated at 10^4 cells per ml into oxide media at two selected levels of P (Table 1).

RESULTS AND DISCUSSION

Concentration of P in solution depended on the amount added with the iron oxide (Fig. 1). The minimal measured concentration, 0.05 μM, can be attributed to desorption of endogenous P from untreated limonite, and possible error due to Si.

Phosphate was buffered against depletion by growing bacteria until population density exceeded 10^6 cells per ml (Fig. 2). Buffering was less effective at low P than at high P, but was still sufficient for determination of P-dependent growth in experiments lasting 2 to 3 days when initial inoculum levels were low (10^3 to 10^4 cells per ml). Nephelometry would be too insensitive for enumeration at these levels; drop-

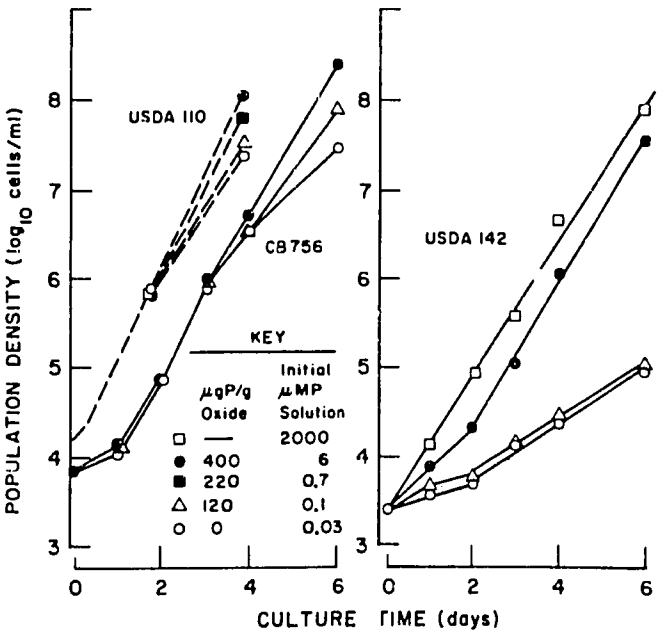

Fig. 3—Effect of P concentration on growth curves for two P-efficient strains *(left)* and an inefficient strain *(right)*. Note lack of P-response by P-efficient strains USDA110 and CB756 until large population caused P-demand to exceed buffering of the culture system at 3 to 4 days.

counting was convenient, precise, and free of uncertainties about dead cells and effects of strain, treatment, and gum production on calibration factors [P nutrition has large effects on gumminess and cell length in some strains, not others (2)].

Spurious growth responses developed when cell population exceeded the culture system's buffering capacity. The differences that became apparent after day 4 (Fig. 3), with more than 10^7 cells per ml, simply reflect differences in release rate from the oxide depending on differences in the amount of sorbed P. They were not due to exhaustion of the reserve of sorbed P. This was checked by analysis of cells from cultures at 400-μg P/g oxide. They contained 1.5% P (dry weight). In a 100-ml culture with 10^7 cells per ml this amounts to < 2 μg P, and is negligible in relation to the adsorbed reserve even at the low level of 50 μg/g (150 μg/culture).

With P concentrations under control, before day 4, strain CB 756 and USDA 110 grew at identical rates at all P concentrations tested (Fig. 3); and as rapidly as in conventional media (5). By contrast, from the beginning, strain USDA 142 grew slowly at low P concentrations. This comparison establishes that strains differ markedly in external P requirements for growth.

Earlier work (2) has shown that *Rhizobium* grown in media at conventional "high" P concentration (2,000 μM) stored P sufficient to support 3 to 5 generations in unbuffered low-P medium. To eliminate storage effects, all cultures in the present experiments were inoculated with P-depleted rhizobia. Strains differed in their ability to store P at high external concentration (2), as well as the ability to grow at low external P concentration. Both properties might be important in adaptation to natural varying P environments.

A further screening of six strains of *R. japonicum* (Table 1) indicates a range of variation in sensitivity to low P. These six rhizobia grow at similar rates at 2,000 μM P. Their ranking in Table 1 resembles their ranking in ability to nodulate and to establish early nitrogen fixation on Clark soybean in low-P sand culture (Cassman and Munns, in preparation), as well as their ability to accumulate stored P when grown in high-P media (2).

The strain differences seem large enough to be agronomically significant. These differences might be one reason for conflict between reports on effects of P on legume nodulation. The efficient strains USDA 110 and CB 756 should tolerate P deficiency in the field. Indeed, both are successful commercial strains. By contrast, strains as sensitive as USDA 142 might

Table 1—Growth of six *R. japonicum* strains in a limonite dialysis-culture medium buffered at two phosphorus concentrations.

Strain	Initial cell density	Generations after 76 hours growth	
		$4\times10^{-7}M$ P	$6\times10^{-6}M$ P
	Cells per ml	number	
USDA 138	940	6.9*	9.3
USDA 110	910	6.4	6.5
USDA 6	840	5.3	8.4
USDA 136	1,220	4.8	5.7
USDA 122	1,280	4.6	5.6
USDA 142	1,170	2.4	3.9

* L.S.D. ($p = 0.05$) for comparison of means of one strain at different P levels or strain differences at the same P level are 0.5 and 0.3, respectively.

do well in artificial media in greenhouses or growth chambers but fail to grow in a normally P-depleted rhizosphere in soil. Screening out sensitive strains would save the expense of testing them in soil or the greater expense of innocently taking them to field inoculation trials. For this purpose, our procedure could be simplified: two or three counts at one selected P level might be sufficient.

The iron oxide dialysis culture method may lend well to studies involving P-limitation of other microorganisms. For research with P-efficient organisms, such as the more efficient strains in this study, our procedure needs modification to improve buffering and monitoring capability at concentrations well below 0.1 μM. These modifications could include the use of synthetic oxide with high reactivity and little endogenous P; the use of more oxide or a larger dialysis surface, depending on whether desorption or membrane diffusion is rate-limiting; and improved analytical sensitivity.

ACKNOWLEDGMENTS

We are grateful to Kim Clarkin for her enlightening struggles with unsuccessful early versions of the procedure; to Lynn D. Whittig for X-ray analysis; and to Harold Keyser (USDA) and Patricia Nakao (NifTAL) for the rhizobia. This work was supported by a grant from NSF/RANN and a subcontract from AID/University of Hawaii NifTAL Project.

LITERATURE CITED

1. Andrew, C. S., and E. J. Kamprath. 1978. Mineral nutrition of legumes in tropical and subtropical soils. C.S.I.R.O., Melbourne, Aust.
2. Cassman, K. G., D. N. Munns, and D. P. Beck. 1981. Phosphorus nutrition of *Rhizobium japonicum*: strain differences in phosphate storage and utilization. Soil Sci. Soc. Am. J. 45:517–520 (this issue).
3. Gates, C. T. 1974. Nodule and plant development in *Stylosanthes humilis*. Aust. J. Bot. 22:45–55.
4. Helyar, K. R., and D. N. Munns. 1976. Phosphate fluxes in the soil-plant system: a computer simulation. Hilgardia 43:103–130.
5. Keyser, H. H., and D. N. Munns. 1979. Tolerance of rhizobia to acidity, aluminum and phosphate. Soil Sci. Soc. Am. J. 43:519–522.
6. Nye, P. H. 1979. Soil properties controlling the supply of nutrients to root surfaces. p. 21–38. *In* J. L. Harley and R. S. Russel (ed.) The Soil-root interface. Academic Press, London.
7. Reisenauer, H. M. 1966. Mineral nutrients in soil solution. p. 507–508. *In* P. L. Altman and D. S. Dittman (ed.) Environ. Biol. Fed. Am. Soc. for Exp. Biol., Bethesda, Md.
8. Throneberry, G. O. 1974. Phosphorus and Zn measurements in Kjeldahl digests. Anal. Biochem. 60:358–362.
9. Vincent, J. M. 1970. A Manual for the practical study of root nodule bacteria. Blackwell, Oxford.
10. Werner, D., and K. Berghauser. 1976. Discrimination of *Rhizobium lupini*, *R. trifolii*, *R. leguminosarum*, and of bacteroids by uptake of 2-ketoglutaric acid, glutamic acid, and phosphate. Arch. Microbiol. 107:257–262.