APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1980, p. 466-469
0099-2240/80/02-0466/04$02.00/0

Vol. 39, No. 2

Density Centrifugation Method for Recovering *Rhizobium* spp. from Soil for Fluorescent-Antibody Studies†

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A density centrifugation procedure has been developed as a replacement for soil flocculation and clarification steps employed in quantitative fluorescent-antibody studies on *Rhizobium* in soils. Near-quantitative recovery of added cells of two strains of *Rhizobium japonicum* and two strains of *R. phaseoli* was achieved from six soils with various properties. It is proposed that this technique may prove useful in separating other soil microorganisms from soil particles in ecological studies employing fluorescent-antibody techniques.

Fluorescent-antibody (FA) techniques have provided a new and useful approach for autecological studies of microorganisms in natural environments (1, 3, 6, 8). A significant improvement in these techniques was the development of quantitative FA procedures (7).

However, C. Vidor and R. H. Miller (Soil Biol. Biochem., in press) reported recoveries of 20% of an added *Rhizobium japonicum* strain in one soil, and 80% in another. Donaldson et al. (M. D. Donaldson, D. F. Bezdicek, and B. Sharma, Proc. 7th N. Am. Rhizobium Conf., p. 5, 1979) reported average recoveries of 44% for *R. japonicum* and 33% for fast-growing rhizobia. Robert and Schmidt (F. M. Robert and E. L. Schmidt, Proc. 7th N. Am. Rhizobium Conf., p. 7, 1979) reported recoveries in the same range for cells of *R. phaseoli*. Kingsley and Bohlool (M. R. Kingsley and B. Bohlool, Proc. 7th N. Am. Rhizobium Conf., p. 8, 1979) found recoveries of chickpea rhizobia from tropical soils to be approximately 1%, although 100% recovery was obtained from one inceptisol.

The primary reason for reduced cell recoveries in FA procedures is that flocculation of soil colloids necessary to provide a clarified supernatant which can be effectively filtered may cause the coflocculation of microbial cells. In addition, we found that it was particularly difficult and time consuming to clarify and filter supernatants from soils containing large quantities of silt-sized quartz particles which possessed little electrical charge.

The desirability of improving the recovery of *Rhizobium* cells from soil as well as the possibility of decreasing the time and complexity of the quantitative FA procedures were the impetus for this study. A report on the utilization of zonal centrifugation to isopycnically band soil clay minerals (4) led to our evaluation of density centrifugation for sample preparation for FA analysis and is the subject of this report.

The A horizons of six soils representing four soil orders were selected for use (Table 1). These soils differed greatly in properties such as soil texture, mineralogy, pH, and cation exchange capacity.

R. japonicum strain 587 was used for most studies. This organism originated in Brazil and came from the culture collection of C. Vidor. Serogroup 110 of *R. japonicum* originated from the Beltsville, Md., collection and was supplied by D. Weber. Serogroups 442 and QA 1062 of *R. phaseoli* were two accessions to the culture collection of R. H. Miller and originated in Brazil and Colombia, respectively.

Since recovery percentages could change with the phase of growth, cells were always grown to about the mid-log phase. *Rhizobium japonicum* strains were grown in sodium gluconate broth (2) on a rotary shaker at 28°C. At that time the medium contained about 5×10^8 cells/ml as determined by plate counts, direct counts using a Petroff-Hausser counting chamber, or immunofluorescence. *R. phaseoli* were grown in soil extract, yeast mannitol broth to about 10^8 cells/ml as determined by direct counts.

Field moist samples were used, or dry samples were premoistened with water and allowed to equilibrate for 12 to 18 h before addition of the organisms. Inocula were added to bring the soil moisture to a water potential of about −0.3 bars. About 10^8 rhizobia/g of soil were added, unless otherwise specified. Normally at this level, sample dilution, even without clarification by floc-

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TABLE 1. *Selected soil properties of experimental soils*

Soil type and origin	Soil order	Acidity (pH)	Cation exchange capacity (meq per 100 g of soil)	Organic C (%)
Lynchburg sandy loam (N. Carolina)	Ultisol	6.0	3.0	2.8
Rossmoyne silt loam (Ohio)	Alfisol	6.5	6.0	2.2
Miamian silt loam (Ohio)	Alfisol	7.3	8.0	1.8
Brookston silty clay loam (Ohio)	Mollisol	6.8	21.0	5.8
Iracema (dusky red latosol) clay (Brazil)	Oxisol	5.1	16.8	1.8
Cerrado (dark red latosol) clay (Brazil)	Oxisol	4.9	1.5	1.7

culation, would probably permit effective filtration; however, cell density was chosen, independent of the need for flocculation. Its selection was based, rather, on the requirement to challenge the efficacy of the centrifugation step of the proposed procedure. Samples requiring incubation were held at ambient temperatures, usually 24 ± 2°C, and lost moisture was replenished about every 48 to 72 h by additions of distilled water.

To evaluate rhizobial recovery, individual 10-g soil samples were quantitatively transferred to a stainless-steel blender cup with 95 ml of a 1% solution of $CaCl_2$. One drop (Pasteur pipette) of Tween 80 (Difco Laboratories) and three drops (Pasteur pipette) of an antifoam agent (Dow-B, Fisher Scientific Co.) were added to the soil suspension. The suspension was blended for 5 min on a multimixer (Prince Castle Manufacturing Div., Inc., Sterling, Ill.), after which a 10-ml portion was withdrawn and placed in 20 ml of a 1.33-g/cm^3 sucrose solution contained in a 100-ml clear polypropylene centrifuge tube. The soil suspension-sucrose solution was thoroughly mixed, and 30 ml of a 1.33-g/cm^3 sucrose solution was layered beneath the soil suspension.

Samples were generally centrifuged at 715 × *g* for 15 min, using an International Equipment Corp. centrifuge with a swinging bucket rotor with a capacity for eight 100-ml centrifuge tubes. We observed that the Miamian and Brookston soils were satisfactorily clarified at 252 × *g* and 715 × *g*, whereas the Rossmoyne and Lynchburg soils were clarified only at 715 × *g*. A centrifugal force of 1,479 × *g* reduced recovery of *R. japonicum* strain 587 by over 1 log. The two Oxisols caused the greatest problems for clarification, and additional studies should be conducted to evaluate a greater range of centrifugal force or centrifugation times or both.

After centrifugation, 10-ml portions were withdrawn from the sucrose layer and suspended in 90 ml of distilled water. Additional decimal dilutions were made as necessary, which depended on the expected number of cells in the original soil sample. Portions were withdrawn from the last dilution for immunofluorescent analysis.

Nuclepore filters (25 mm, 0.4 μm) were used. These filters were soaked overnight in a dilute solution of surfactant (one drop of Tween 30 in about 20 ml of distilled water) to overcome the hydrophobic properties of the filter (5).

Instead of following the method of Schmidt (7) for drying rhodamine gelatin to reduce nonspecific adsorption of FA-labeled antibodies, we left filters in place on the filter holder. Then six drops of water (Pasteur pipette) were added to the filter followed by three drops of rhodamine-conjugated gelatin. The entire filter holder was removed from the filter flask and gently shaken to uniformly moisten the entire filter surface with the rhodamine gelatin. After returning the assembly to the suction flask, a vacuum was applied momentarily to remove excess rhodamine gelatin. The tip of the filter holder was sealed with parafilm, and the filter (still in place) was stained with six to eight drops (Pasteur pipette) of the appropriate fluorescein isothiocyanate-conjugated antiserum. The entire filter assembly was incubated in the dark for 30 min. Subsequently, the filters were washed with at least 250 ml of physiological saline.

To assure a dark background, Nuclepore filters were positioned on a microscope slide directly over an India ink-stained membrane filter (25-mm diameter, 0.45 μm; HABG, Millipore Corp.) and treated with mounting fluid (Difco). This simple procedure proved highly effective in providing the desired contrast for microscopic enumeration and reduced the necessity of the more difficult blackening of the Nuclepore filter. The Nuclepore filters increased FA counts of *R. japonicum* strain 587 by about 0.5 log in comparative studies with Millipore filters.

Each filter was covered with a glass cover slip and examined by using a Leitz Ortholux microscope equipped for epifluorescent microscopy. Samples on Nuclepore filters were held for as long as 18 h without apparent loss of counting efficiency. Counts were based on an average of 20 fields per slide, selected at random, which

provided a relatively constant coefficient of variability for counting with soils of comparable cell densities (Vidor and Miller, Soil Biol. Biochem., in press).

The mean recovery of *R. japonicum* strain 587 from the six soils averaged 97%, with a range of 89 to 107% (Table 2). The lower recovery of cells from Rossmoyne soil as compared with other soils was the result of a 78% recovery in one of three separate studies. Recoveries in two other studies with this soil exceeded 90% of the cells added. These recoveries by density centrifugation are certainly manifold better than the recoveries achieved with clarification by flocculation (Donaldson et al., Proc. 7th N. Am. Rhizobium Conf., 1979; Robert and Schmidt, Proc. 7th N. Am. Rhizobium Conf., 1979; Vidor and Miller, Soil Biol. Biochem., in press). For strain 587, at least, there appeared to be no relation between soil properties and recovery.

As an additional test of the efficacy of the procedure, two different rhizobial species were added to soils. For *R. japonicum* strain 110, 116% of the added cells were recovered from Lynchburg soil. Since strain 110 produces copious quantities of extracellular polysaccharide, there would be ample opportunity for the extracellular polysaccharide to interact with the soil constituents and cause recovery problems. This did not occur.

The recovery of *R. phaseoli* strains 442 and QA 1062 from the Rossmoyne soil was 110 and 104%, respectively, and 99% for strain QA 1062 from the Iracema soil. However, only 44% of the added strain 442 cells were recovered from Iracema soil. Although this recovery value is low in comparison with others reported in this study, it is comparable to and in some cases better than cell recoveries reported by using the soil flocculation procedure for clarifying soil suspensions as a preparative step for FA analysis of soil organisms.

TABLE 2. *Recovery of R. japonicum strain 587 from six different soils, using density centrifugation for clarification and FA counting*

Soil	Recovery[a] (%)
Lynchburg	96[b]
Rossmoyne	89[c]
Miamian	107[d]
Brookston	100[c]
Iracema	107[d]
Cerrado	103[d]
Mean	97

[a] Recovery based on numbers of cells added to soil as determined by direct counts (Petroff-Hausser or FA).
[b] Mean of four recovery experiments.
[c] Mean of three recovery experiments.
[d] Single recovery experiment.

Over the range of 10^6 to 10^9 cells of strain 587 added to soil, the recovery averaged 99% and ranged from 89 to 102%. The percent recovery at the highest density was not significantly different from the lowest. Recoveries of *Rhizobium* populations of less than 10^6/g of soil were not attempted, although Bezdicek (personal communication) has quantitatively recovered about 10^5 rhizobia/g of soil by using a similar procedure to ours. Additional modifications and refinements should permit good recoveries at even lower population levels.

Preliminary data also indicate that recoveries of *R. japonicum* strain 587 could be affected by the soil moisture content at the time the cells were added to the soil. *Rhizobium* cells added to the two Oxisols (premoistened to a water potential of approximately −0.3 bars) were recovered quantitatively. When a comparable number of cells was added to these same soils in an air-dried state, only 80% of the added cells were recovered. These data may be explainable on the basis of differing cell adsorption on soil colloids with differing water film thickness or perhaps cell destruction due to localized heat of wetting or membrane breakage or both during soil wetting.

The earlier recovery studies were based on the addition of rhizobia cells and immediate extraction and enumeration. Therefore, two additional studies were performed to evaluate the survival and recovery of *R. japonicum* strain 587 from soils over longer time periods. These data are reported in Fig. 1A and B. Fig. 1A shows that recoveries on days 1 and 4 on the Rossmoyne and Lynchburg soils were nearly identical to those on day 0. The recovery at day 0 was 94% of the cells which had been added. After day 4, cell numbers declined until slightly more than a 1-log decrease occurred at day 11. These data could be explained as either the death of the cells or as decreased efficiency in cell recovery. The former explanation seems more correct since similar survival curves from Rossmoyne soil were obtained by viable counts for *R. japonicum* (Vidor and Miller, Soil Biol. Biochem., in press). Recoveries of *R. japonicum* strain 587 from the two Oxisols were similar for the 4-day period (Fig. 1B). The value at day 0 represents 104% of the cells added. These data certainly suggest that the density centrifugation method effectively removes *Rhizobium* cells from soil colloids after periods of interaction with soil up to 4 days.

This note reports data evaluating density centrifugation as a method for recovering two species and four strains of *Rhizobium* from soils for

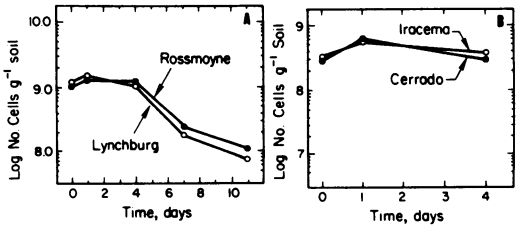

FIG. 1. *(A) Survival of R. japonicum strain 587 added to Rossmoyne and Lynchburg and (B) to Iracema and Cerrado soils, using density centrifugation for clarification and FA counting.*

quantitative FA enumeration. Much more extensive evaluation of the method is needed to verify its broad applicability to other *Rhizobium* spp., other soils, and a range of other important soil microorganisms.

This study was supported in part by U.S. Department of Agriculture CR contract no. 701-15-20. We thank J. Bigham, Dept. of Agronomy, Ohio State University, for supplying the Brazilian Oxisols used in this study.

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