Reprinted from the *Soil Science Society of America Journal*
Volume 46, no. 3, May-June 1982
677 South Segoe Rd., Madison, WI 53711 USA

Effect of Suspending Agent and Temperature on Survival of *Rhizobium* in Fertilizer[1]

R. J. KREMER, J. POLO, AND H. L. PETERSON[2]

ABSTRACT

The lack of compatibility between legume inoculants and fertilizers has been widely recognized and reported. Studies involving alternative inoculant carriers and methods of inoculant preparation led to an examination of rhizobia incorporation in fertilizer. The effects of suspending agent and incubation temperature on the survival of two strains of *Rhizobium* in 0-9-12 fertilizer were determined using plate counts and most-probable-number methods of analyses. Results showed that oil was superior to water in maintaining the viability and effectiveness of two strains of lyophilized rhizobia. A fertilizer incorporated with lyophilized *R. phaseoli* suspended in oil contained nearly 10^4 viable cells per gram after 24 weeks of incubation at 65°C. In contrast, no *R. phaseoli* were recovered from a fertilizer containing a water suspension of lyophilized cells after 8 weeks of incubation at 25°C. These results strongly suggest that fertilizer and lyophilized rhizobia may be combined to form an effective inoculant if adequate precautions are taken to protect the rhizobia from rehydration.

Additional Index Words: inoculant-containing fertilizer, legume inoculation, lyophilized rhizobia, rhizobial survival.

Kremer, R. J., J. Polo, and H. L. Peterson. 1982. Effect of suspending agent and temperature on survival of *Rhizobium* in fertilizer. Soil Sci. Soc. Am. J. 46:539–542.

LEGUME inoculants are often subjected to adverse environmental conditions during storage, shipping, and planting which may result in decreased viability of rhizobia and poor nodulation of the host legume. Various inoculant carrier formulations have been developed to enhance rhizobial survival (16). Peat-base inoculants, however, are considered superior in the ability to deliver large numbers of viable rhizobia at seed inoculation (6, 20).

Incorporation of inoculants in various pelleting materials reportedly prolongs the survival of rhizobia when applied to legume seed (3, 4, 5). These materials include many forms of calcium carbonate, dolomite, gypsum, superphosphate, and rock phosphates (3, 4, 13, 14, 16, 17). The wide variations reported for survival of rhizobia in different pelleting materials (3, 17) has led to recommendations that sources of pelleting materials be thoroughly screened for effects on rhizobial viability before use in seed pelleting (14, 17).

While there are few reports concerning incorporation of rhizobia in fertilizers, the practice of mixing inoculants directly with fertilizers has been discouraged. Burton and Curley (7) found that sodium molybdate reduced numbers of *R. japonicum* 99% after 4 days of incubation. Various molybdenum compounds mixed with peat-base soybean inoculant and stored prior to seed treatment significantly reduced nodulation (12). Studies examining the effect of different fertilizer concentrations on crown vetch rhizobia in slurry mixtures showed that the rhizobia survived only when the slurry pH was raised to 6.0 with $CaCO_3$ (6). Fraser (11) used calcium sulfate as a carrier for rhizobia and prepared granules containing 10^7 *R. meliloti* per gram. After 5 months, granules stored at 25°C still contained 10^5 viable rhizobia per gram. Carr and Ballard (9) mixed a *R. trifolii* inoculant with solutions of potassium chloride and 10-30-10 fertilizer (90 kg/m^3) for 1 hour and reported a slight increase in numbers of rhizobia.

Survival studies with *Rhizobium* in fertilizers and pelleting materials have involved the use of liquid or peat-base cultures of rhizobia. Death of *Rhizobium* in these cultures occurs rapidly at high temperatures due to desiccation (6, 18, 19, 21) and with fertilizers due to pH and osmotic effects (6, 21). Lyophilized rhizobia used in some inoculants maintain high viability over extended periods of storage (2, 10, 15, 16, 23). Increased survival rates at 28 to 36°C exhibited by lyophilized rhizobia compared to peat-base cultures are due to increased resistance to desiccation (21, 22). There is no published information regarding the compatibility of lyophilized rhizobia with fertilizers. The detrimental effects of fertilizers on rhizobia might be partially overcome by using lyophilized cultures suspended in a nonaqueous system. This paper summarizes an investigation of the feasibility of using oil as a suspending agent in combining *Rhizobium* and fertilizer.

MATERIALS AND METHODS

Strain CA22 of peanut *Rhizobium* and *R. phaseoli* 971A were grown in yeast extract-mannitol (YEM) broth (23) shaker cultures at 25°C. The cultures were harvested after 4 days of growth for *R. phaseoli* 971A and 10 days for peanut *Rhizobium* CA22 by centrifugation at 1,800 × g for 60 minutes at 4°C. The pellets were resuspended in phosphate-buffered saline (PBS: 10 m*M* K_2HPO_4–KH_2PO_4, 0.14*M* NaCl, pH 7.2) and centrifuged. The washed pellets were suspended in minimal amounts of a solution composed of 7.5% sucrose, 5.0% dextran, and 1.0% sodium glutamate, transferred to sterile plastic petri dishes, quick-frozen in liquid nitrogen, and freeze-dried for 48 hours.

Lyophilized cells were dispensed into bags of autoclaved oven film (Reynolds) and the bags were heat-sealed. The bags were placed in polyethylene packages containing $CaSO_4$ as desiccant and stored at 8°C. The final concentration of lyophilized rhizobia was 5.0×10^{10} cells per gram for *R. phaseoli* 971A and 3.8×10^{10} cells per gram for peanut *Rhizobium* CA22.

Lyophilized rhizobia were suspended in peanut oil (10 g/200 ml) or filter-sterilized deionized water (10 g/150 ml) as a control. Peanut oil was previously rendered free of rhizobia and water by heating at 120°C for 12 hours. Suspended rhizobia were added slowly to 1 kg of 0-9-12 fertilizer. The 0-9-12 fertilizer (pH 7.48) was an ungranulated mixture of 100 g of KCl, 170 g of KH_2PO_4, 320 g of rock phosphate (34% P_2O_5), and 410 g of $CaCO_3$. Fertilizer and inoculant were mixed using a household mixer. Additional amounts of water or oil were added to fertilizer to quantitatively transfer and incorporate the inoculant. Rhizobia-fertilizer

[1] Authorized as Paper no. 4,296 in the Journal Series of the Mississippi Agricultural and Forestry Experiment Station. This research was conducted under project AG/TAB 610-9-76 and partially supported by USDA/SEA/CR/USAID Competitive Grant 616-15-188. Received 9 Feb. 1981. Approved 11 Jan. 1982.

[2] Research Associate, Mississippi State Univ., Mississippi State; Chemical Engineer, International Fertilizer Development Center, Muscle Shoals, Ala.; and Associate Professor of Agronomy, Mississippi State Univ.

mixtures were placed in a 40 cm in diameter pan granulator and rotated at 40 to 50 rpm at a 45° angle for about 45 minutes. All equipment used in inoculant preparation and fertilizer granulation was washed in 5.25% sodium hypochlorite (Clorox) and rinsed with sterile water before use.

Each fertilizer treatment was packaged as 10 g (dry weight) in ethylene oxide-sterilized polyethylene bags and heat-sealed. Analyses for initial numbers of rhizobia in the fertilizers were made 2 days after mixing the fertilizer and rhizobia. Fertilizer containing rhizobia was stored at 5°C prior to initial analyses and initiation of the incubation study. Samples were then incubated at 25, 45, or 65°C. Duplicate bags of each treatment × temperature combination were removed for analyses at specified sampling dates. The 10-g sample was suspended in 90 ml of a solution composed of 0.1% peptone, 2% Span 85, and 2% Tween 85 (ICI America, Inc.) and shaken for 15 minutes. Serial 10-fold dilutions were made in 0.1% peptone water, and viable numbers of rhizobia were determined by spread-plating on duplicate plates of YEM agar containing 2.0 ppm brilliant green to suppress the growth of contaminants. Colonies were counted after incubation at 25°C for 6 days with *R. phaseoli* 971A and 10 days with peanut *Rhizobium* CA22.

Numbers of nodule-forming rhizobia were determined by the most-probable-number (MPN) technique (23). One-milliliter aliquots of serial dilutions of fertilizer containing *R. phaseoli* 971A were pipetted onto rhizobia-free seeds of *Phaseolus vulgaris* L. cv. 'Commodore' in plastic growth pouches supported in record racks (24). Four pouches containing two seeds each were inoculated with each dilution. Uninoculated seeds served as controls. Plants were grown in a growth chamber maintained at 28°C during a 16-hour-light period and 19°C during an 8-hour-dark period. After 4 weeks of growth, plants were examined for nodulation, and MPN values were calculated. In a similar manner, rhizobia-free seeds of *Arachis hypogaea* L. cv. 'Florunner' were inoculated with dilutions of fertilizer-containing peanut *Rhizobium* CA22. The peanut seeds were planted in 473-cm^3 (1 pint) pots containing an autoclaved mixture of 2 parts river sand: 1 part vermiculite: 1 part perlite with pH adjusted to 6.5 with lime. Four pots containing two seeds each were inoculated with each dilution. The seeds were covered with 2 cm of the sterile potting mixture and transferred to the greenhouse. After 6 weeks of growth, plants were removed from the containers, roots were washed free of potting mixture, nodulation noted, and MPN values determined.

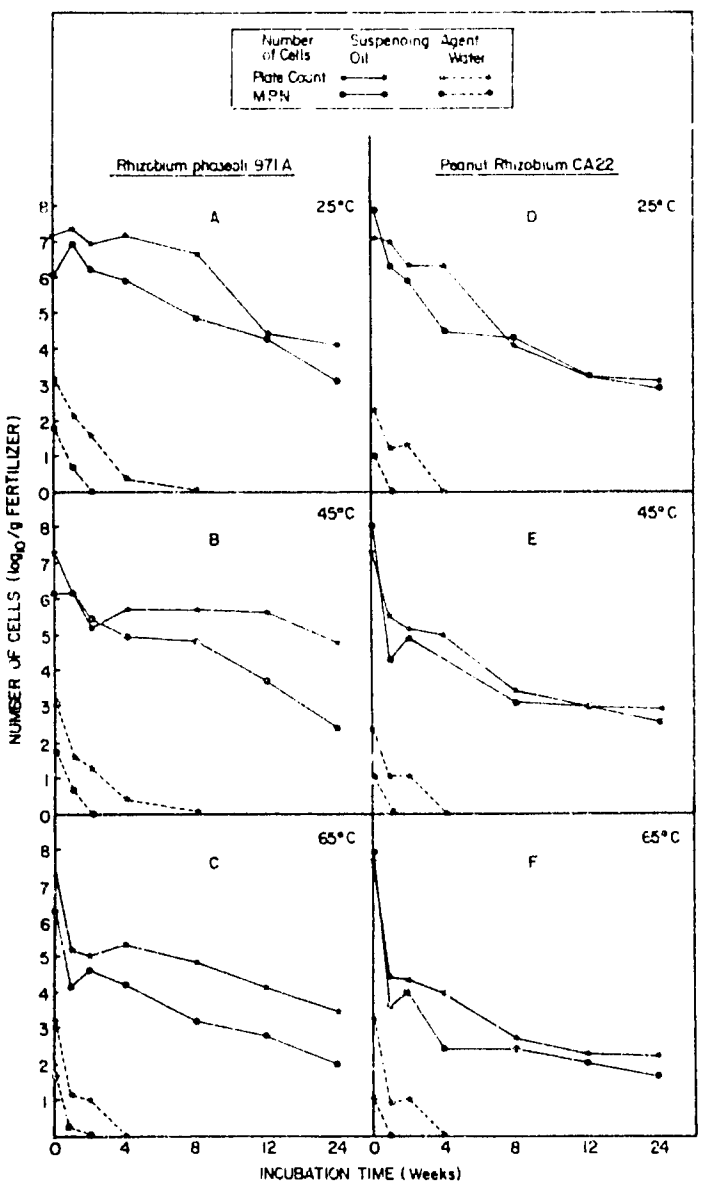

Fig. 1—Effect of suspending agent and temperature on survival of *Rhizobium phaseoli* 971A and peanut *Rhizobium* CA22 incorporated in 0-9-12 fertilizer. L.S.D.$_{0.05}$ between plate counts 1.45 and 1.85, respectively, for *R. phaseoli* 971A and *Rhizobium* CA22. L.S.D.$_{0.05}$ between MPNs 1.14 and 0.57, respectively, for *R. phaseoli* 971A and *Rhizobium* CA22.

RESULTS

Stable free-flowing granules were formed when rhizobia suspended in water were mixed with fertilizer. Large fertilizer granules, 5 to 10 mm in diameter, were formed when 190 ml of water containing *R. phaseoli* was mixed with 1 kg of fertilizer. Smaller granules (1 to 5 mm) were formed when 170 ml of the *Rhizobium* CA22 suspension was added to 1 kg of fertilizer. Granules prepared with rhizobia suspended in oil were not uniform, very fragile, and tended to aggregate in the package. Fertilizer (1 kg) mixed with 290 ml of *Rhizobium* CA22 in oil formed fragile, individual granules. Much larger, massive aggregates formed when 300 ml of *R. phaseoli* 971A in oil was combined with 1 kg of fertilizer. Packages containing the inoculant mixtures remained intact after 24 weeks of incubation at all temperatures. Granules prepared with water remained free-flowing and free from aggregates, while those prepared with oil became slightly aggregated.

The survival of *R. phaseoli* 971A and peanut *Rhizobium* CA22 in fertilizer over a 24-week period is presented in Fig. 1. Viable numbers (plate count) of *R. phaseoli* 971A suspended in oil remained fairly constant at 10^7 cells per gram for 8 weeks and declined slightly after 12 weeks of incubation at 25°C (Fig. 1A). The most-probable-number analysis revealed a similar survival trend. Nodules formed on bean plants were large, located on the upper root system, and they had pink to red interiors, indicating the presence of leghaemoglobin (23). In contrast, plate counts of *R. phaseoli* 971A suspended in water and incorporated in fertilizer declined rapidly during incubation. No viable rhizobia were detected after 4 weeks. Also most probable numbers of < 100 per gram were obtained for fertilizer initially and were detected only during the first week of incubation. Nodules on bean plants in this MPN analysis were small, scattered on the distal lateral roots, and were white inside. Both plate counts

and MPNs of *R. phaseoli* 971A suspended in oil declined sharply in fertilizer throughout the first 2 weeks of incubation at 45 and 65°C (Fig. 1B and 1C). Thereafter, numbers of rhizobia declined at a much slower rate at both temperatures. Incubation of fertilizer containing water-suspended *R. phaseoli* 971A at 45 and 65°C resulted in a drastic decline in plate counts and MPNs; no viable cells were detected after 4 weeks. Nodulation of plants inoculated with inoculant-containing fertilizer incubated at 45 and 65°C was similar to the respective samples incubated at 25°C.

Survival trends and nodulation patterns for peanut *Rhizobium* CA22 in fertilizer at all incubation temperatures (Fig. 1D-F) were analogous to those observed for *R. phaseoli* 971A. A greater decline in numbers of oil-suspended rhizobia (plate count and MPN) was observed for *Rhizobium* CA22 than for *R. phaseoli* 971A at all temperatures over the first 12 weeks. Greater survival of the fast-growing *R. phaseoli* 971A relative to the slow-growing *Rhizobium* CA22 may be due to differential response of these bacteria to lyophilization and suspension in oil and to exposure to high temperatures.

Previous work (8) has demonstrated that a high molecular weight polymer of polyethylene glycol (PEG 6000) protected fast-growing rhizobia from desiccation better than slow-growing rhizobia. The detrimental effects of fertilizer and high temperature on viability of *Rhizobium* CA22 suspended in water were similar to those observed for *R. phaseoli* 971A.

DISCUSSION

Initial numbers of both *Rhizobium* species in fertilizer were about 10^7 cells per gram with oil and about 10^3 cells per gram with the water control. These numbers were less than the expected 10^8 cells per gram. A partial explanation of the discrepancy for the oil carrier may be the heterogenous mixture of lyophilized cells in oil. Uneven mixing could have resulted in some fertilizer granules containing more cells than others. Factors such as incomplete transfer of oil suspensions to the fertilizer mixture and loss of numbers during the granulation process may have contributed to initial decreases in rhizobial numbers. Techniques are being developed to improve homogenization of lyophilized rhizobia-oil suspensions and to increase granulation efficiency.

Greater survival of lyophilized rhizobia in fertilizer with oil suspensions compared to the water control at temperatures of 25 to 65°C was apparently due to protective effects of the oil. Examinations with the aid of phase microscopy revealed that many of the rhizobial cells were dispersed individually throughout the oil suspensions. This suggests that cells are enclosed individually in an oil film. The oil film may protect rhizobia against toxic effects of fertilizer salts. Poor survival of water-suspended rhizobia in fertilizer was probably due to direct contact with high salt concentrations. Detrimental effects of fertilizers in contact with unprotected rhizobia are well documented (5, 21, 22).

Plate count and MPN values were closely correlated (r = 0.87) for rhizobia in fertilizer granules prepared with oil-suspended cells. Apparently, oil allowed rhizobia to maintain their infective and effective traits despite being exposed to fertilizer salts and high temperatures. This is in sharp contrast to the almost immediate loss of infectiveness for rhizobia when water was used as the suspending agent. The most-probable-number technique, therefore, provided a reliable check on the infectiveness and effectiveness of the fertilizer-base inoculants as well as an estimation of rhizobial numbers.

Oil enabled rhizobia to survive the stress of temperatures as high as 65°C. Poor survival of rhizobia in peat-base and water-base inocula subjected to periods of high temperature can be attributed to extreme desiccation of the organisms (6, 18, 19, 21, 11). Bushby and Marshall (8) reported that rhizobia were protected against desiccation in dry soil when sugars or polyvinyl pyrrolidone were mixed with rhizobia prior to incorporation in soil. Oil may perform a similar function in fertilizer. Oil, heat-treated to remove water, may protect lyophilized rhizobia from rehydration during storage and thereby prevent cellular contact with excessive amounts of soluble salts. Lyophilized rhizobia have a low moisture content and are essentially unaffected by desiccation; however, viability may decrease due to other factors such as contact with oxygen or light (22, 23). Improved survival may be inherent with the use of lyophilized rhizobia since this type of culture is more stable during storage at high temperatures than other forms of inocula (15, 21, 22).

Rhizobial survival was determined in fertilizer-inoculant granules with considerable variation in size and structure. Granules formed with aqueous materials can be properly sized through screening and recycling operations on a commercial scale (1). Granulation with oil appears to be a unique approach to fertilizer technology, and additional research on granule stabilization is needed before an oil-base, granular fertilizer can be developed for use in the field.

Results of the present study indicate that oil can promote survival of rhizobia incorporated in an 0-9-12 fertilizer and incubated at high temperatures. The practical advantages of such a fertilizer-base inoculant include delivery of viable and effective rhizobia after shipping and storage under adverse conditions. In terms of use, a fertilizer inoculant would be extremely advantageous in simultaneously supplying plant nutrients and promoting nodulation and nitrogen fixation. Research is in progress to evaluate the performance of the inoculant in association with the host plant and when subjected to different moisture levels and conditions of temperature-moisture stress under greenhouse and field conditions.

REFERENCES

1. Achorn, F. P., and T. R. Cox. 1971. Production, marketing, and use of solid, solution, and suspension fertilizers. p. 381–412. *In* R. A. Olson et al. (ed.) Fertilizer technology and use. Soil Science Society of America, Madison, Wis.
2. Appleman, M. D., and O. H. Sears. 1944. The possibility of using lyophiled cultures as legume inoculants. Soil Sci. Soc. Am. Proc. 9:98–100.
3. Brockwell, J. 1962. Studies on seed pelleting as an aid to legume seed inoculation I. Coating materials, adhesives, and methods of inoculation. Aust. J. Agric. Res. 13:638–649.

4. Brockwell, J., and R. D. B. Whalley. 1970. Studies on seed pelleting as an aid to legume seed inoculation. 2. Survival of *Rhizobium meliloti* applied to medic seed sown into dry soil. Aust. J. Exp. Agric. Animal Husb. 10:455–459.
5. Burton, J. C. 1975. Methods of inoculating seeds and their effect on survival of rhizobia. p. 175–189. *In* P. S. Nutman (ed.) Symbiotic nitrogen fixation in plants. Cambridge Univ. Press, Cambridge, England.
6. Burton, J. C. 1976. Some practical aspects of legume inoculation. p. 51–68. Proc. of Winter Meeting of Mississippi Section of American Society of Agronomy, Jackson, Miss., 15 Jan. 1976. Mississippi Section of ASA.
7. Burton, J. C., and R. L. Curley. 1966. Compatibility of *Rhizobium japonicum* and sodium molybdate when combined in a peat carrier medium. Agron. J. 58:327–330.
8. Bushby, H. V. A., and K. C. Marshall. 1977. Some factors affecting the survival of root-nodule bacteria on desiccation. Soil Biol. Biochem. 9:143–147.
9. Carr, W. W., and T. M. Ballard. 1979. Effects of fertilizer salt concentration on viability of seed and *Rhizobium* used for hydroseeding. Can. J. Bot. 56:701–704.
10. Davis, R. J. 1963. Viability and behavior of lyophilized cultures after storage for twenty-one years. J. Bacteriol. 85:486–487.
11. Fraser, M. E. 1966. Pre-inoculation of lucerne seed. J. Appl Bacteriol. 29:587–595.
12. Giddens, J. 1964. Effect of adding molybdenum compounds to soybean inoculant. Agron. J. 56:362–363.
13. Hastings, A., and A. D. Drake. 1960. Inoculation and pelleting of clover seed. N.Z. J. Agric. 101:619–621.
14. McGuire, W. S., M. D. Dawson, and F. C. Crofts. 1978. Effective nodulation and production of subterranean clover with pelleted seeds and small amounts of lime. Oregon Agric. Exp. Stn. Bull. no. 633, Oregon State Univ. Corvallis.
15. McLeod, R. W., and R. J. Roughley. 1961. Freeze-dried cultures as commercial legume inoculants. Aust. J. Exp. Agric. Animal Husb. 1:29–33.
16. Peterson, H. L., and T. E. Loynachan. 1981. The significance and application of *Rhizobium* in agriculture. Int. Rev. Cytol. Suppl. 13:311–331.
17. Radcliffe, J. C., W. S. McGuire, and M. D. Dawson. 1967. Survival of rhizobia on pelleted seeds of *Trifolium subterranean* L. Agron. J. 59:56–58.
18. Roughley, R. J. 1968. Some factors influencing the growth and survival of root nodule bacteria in peat culture. J. Appl. Bacteriol. 31:259–265.
19. Roughley, R. J. 1970. The preparation and use of legume seed inoculants. Plant Soil. 32:675–701.
20. Strijdom, B. W., and C. C. Deschodt. 1975. Carriers of rhizobia and the effects of prior treatment on the survival of rhizobia. p. 151–168. *In* P. S. Nutman (ed.) Symbiotic nitrogen fixation in plants. Cambridge Univ. Press, Cambridge, England.
21. Vincent, J. M. 1958. Survival of the root-nodule bacteria. p. 108–123. *In* E. G. Hallsworth (ed.) Nutrition of the legumes. Academic Press, New York.
22. Vincent, J. M. 1965. Environmental factors in the fixation of nitrogen by the legume. *In* W. V. Bartholomew and F. B. Clark (ed.) Soil nitrogen. Agronomy 10:384–435. American Society of Agronomy, Madison, Wis.
23. Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. IBP Handbk. no 15. Blackwell Science Pub. Oxford, England.
24. Weaver, R. W., and L. R. Frederick. 1972. A new technique for most-probable-number counts of rhizobia. Plant Soil 36:219–222.

41

MEMORANDUM

Date 2 Sep 88

TO: PPC/CDIE/DI, Tina Wilson/Acquisitions - 209 SA-18

FROM: S&T/AGR/RNR, Lloyd R. Frederick

SUBJECT: Reprints of Articles with Partial Support of A.I.D. Contracts/Grants

Attached is one copy of the following article with the supporting contract/grant number:

Kremer, R.J., J. Polo and H.L. Peterson 1982
Effect of suspending agent and Temperature on Survival of Rhizobium in fertilizer.
Soil Sci Soc. Amer. Journ. 46: 539-542

Contract/Grant Number: _____

Project: _____

Attachments: a/s

cc: T.Gill
D. Bathrick

5