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FINAL REPORT

Stability of Effectiveness in Cowpea Rhizobia

Project 801-15-92

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INTRODUCTION

The research results from this project have either been published or are in press as three technical articles in professional journals. Reprints of the articles are attached and constitute the detailed report on the technical accomplishments of the research on this project. Many benefits accrued to the participants of this project besides scientific accomplishments. The benefits are discussed in sections with the two countries as subheadings. The purpose of these sections is to document some of the accomplishments that accrued for each country as a result of project involvement.

Guyana

Mr. Harri Persaud is the principal investigator in Guyana that was involved on this project. He is responsible for all inoculum production and quality control in Guyana. Mr. Persaud was having difficulty in obtaining support for quality control from within his country because resources were very limited and his administration did not recognize the importance of quality control. In conjunction with the project I made two visits to Guyana that were very fruitful in helping Mr. Persaud gain administrative support for his area of responsibility. The administrators within the Ministry of Agriculture made specific commitments to provide facilities to grow plants under controlled conditions for the purpose of inoculant quality control. Our project provided numerous expendable supplies that were used on the project and were essential in maintaining the quality of inoculants.

Through this project Mr. Persaud was able to participate in the VII North American Rhizobium Conference and had the opportunity of meeting with other scientists having similar interests and

responsibilities. Following the conference he spent 3 weeks in my laboratory gaining training in quality control of inoculants and in development of skills needed for participation in our project.

As a result of the project Mr. Persaud recognized the need for examining strains of rhizobia for stability of effectiveness before using them in inoculant production. New strains were provided to him that are now included in the inoculants and will be very instrumental in increasing legume production.

Thailand

Mrs. Yenchi Vasuvat is the principal investigator in Thailand that was involved in this project. She has total responsibility for administration of soil microbiology research in Thailand. Production and distribution of inoculants to farmers is her major responsibility. At the time this project was initiated construction was initiated on a facility to be used for production of inoculants and for research on biological nitrogen fixation. It is the largest government owned and operated inoculant production facility in the world.

Mrs. Yenchi recognized the need for a highly trained individual to operate the facility and direct research on rhizobium. Participation in this project was welcomed because it provided for training an individual that could accept this responsibility and at the same time essential research would be accomplished. Mr. Nantakorn Boonkerd was nominated to participate in the project. He received his Ph.D. as a result of the project and returned to Thailand to assume responsibility for production of inoculants and conduct research on rhizobium.

Through the project Mrs. Yenchi and Dr. Boonkerd were able to participate in the international workshop, held in Cali, Columbia, on

Biological Nitrogen Fixation Technology for Tropical Agriculture. Additionally, Dr. Boonkerd was able to participate in the VII and VIII Rhizobium Conferences. The exposure that they gained through participation in these conferences helped very much in their becoming acquainted with important research directly relevant to Thailand. It also enabled them to make acquaintances that will benefit their programs in the long term.

Through research on the project Dr. Boonkerd learned that stability of effectiveness of rhizobia used in inoculant production in Thailand was very good and that they were well suited to survival in Thailand soil.

CONCLUSION

Participation in this project was very beneficial to all involved. The principal investigators in Guyana and Thailand were provided the opportunity for increased training in nitrogen fixation research and benefited directly from the research results. Principal investigators in both countries gained in obtaining better visibility for their programs within their respective countries and benefited from becoming acquainted with other scientists. The stability of effectiveness and survival of inoculant strains was determined and will be of much importance to the farmers as inoculant usage increases.

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EFFECTIVENESS STABILITY OF COWPEA RHIZOBIA AS AFFECTED BY SOIL TEMPERATURE AND MOISTURE

Nantakorn Boonkerd and R. W. Weaver[1]

ABSTRACT

Inoculation of a soil with rhizobia puts them under various environmental stresses that leads to selection of subpopulations that may not be as effective as the original population. The hypothesis was tested by incubating different strains of rhizobia in a sandy pH 5.7 soil obtained from Thailand. Three strains of cowpea rhizobia were utilized; two nodulated peanuts, Tha-201 and T-1; and one nodulated cowpea, TAL-309. The rhizobia were incubated under dry and moist (-0.33 bar) soil conditions at 40°C in sterilized soil and at 45°C in non-sterilized soil. Eighty single colony isolates of each strain were obtained from nodule isolations of cowpea and peanut which were inoculated with soil samples immediately following soil inoculation and 15 days later for non-sterilized soil. Single colony isolates were also obtained from pour plates of soil dilutions of sterilized soil immediately following inoculation and again after 45 days of incubation. Results indicated that there were changes in effectiveness of some strains due to treatments. Relatively ineffective isolates (<60% of plant dry matter produced as compared to parent culture) were found in relatively high frequency (30% of isolates) in the population of nodule isolates of strain TAL-309. The variation was not as great when strain TAL-309 isolates were obtained directly from soil. Strains Tha-201 and T-1 were comparatively stable in effectiveness. Selection of strains that have stable effectiveness characteristics is important for inoculant production.

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INTRODUCTION

Spontaneous loss of the symbiotic characteristics infectivity and effectiveness of rhizobia have been reported (Roughley, 1976, Schwinghamer, 1964, and Labandera and Vincent, 1975). Frequency of mutation in a single gene, in bacteria is about 10^{-7} and 10^{-8} per cell per generation (Stent and Calendar, 1978). There are a number of genes involved in effectiveness in nitrogen fixation in Rhizobium, including 17 for nitrogenase (Brill, 1980), a hydrogen uptake system (Schubert and Evans, 1976), and symbiosis between legume and rhizobia (Caldwell and Vest, 1977, and Vincent, 1980). Chance for loss of effectiveness by spontaneous mutation is considerable since many genes are involved.

There is also evidence that genes required for nodulation and nitrogen fixation are plasmid-borne in rhizobia (Nuti et al, 1979, Brewin et al, 1980, Buchanan-Wollaston et al, 1980, and Casadesus et al, 1980). Therefore, loss of plasmids from effective rhizobia may also result in ineffective or non-nodulating mutants as was demonstrated by Casadesus et al,(1980).

Variation in symbiotic effectiveness of cowpea rhizobia has been reported by Herridge and Roughley (1975). They found that 17 sub-strains of CB756 varied in symbiotic effectiveness; 9 were fully effective, 5 were moderately effective, 2 were relatively ineffective and 1 was completely ineffective.

The majority of rhizobial isolates obtained from nodules on peanuts in peanut growing areas were either only moderately effective or relatively ineffective (Weaver, 1974). Similar results were obtained with isolates from nodules of clovers (Bergersen, 1970, Bergersen et al, 1971, and Brockwell et al, 1968). Holding and King (1963) and Nutman (1946) reported that soil factors have an influence on variation in effectiveness of rhizobia.

From an economic standpoint ineffective variants in a field environment compete with effective rhizobia for formation of nodules on legumes and decrease the yield potential. The frequency of effective rhizobial strains mutating to become ineffective under stress in the

soil environment has not been adequately examined. The purpose of the experiment reported here was to determine the relative symbiotic stability of various strains of rhizobia to soil temperature and moisture.

MATERIALS AND METHODS

Symbiotic stability of cowpea rhizobia in sterilized and non-sterilized soil conditions was investigated. The soil was a Korat sandy loam (Ustoxic Dystopepts) from Thailand having a pH of 5.7. Three strains of cowpea rhizobia were selected. Two strains were isolated from peanut, Tha-201 (Thailand) and T-1 (Texas A&M University), and were effective on cowpea and peanut. The third strain, TAL-309 was obtained from the NifTAL culture collection, Paia, Hawaii but the original culture designation is CB756 and belonged to the Sydney University collection, New South Wales, Australia and is effective on cowpea. Two moisture regimes, air dry and moist (-0.33 bar) and two temperatures, 40°C for sterilized soil and 45°C for non-sterilized soil, were used for incubations. All treatments were replicated twice. Soil was sterilized by autoclaving 10 g samples in test tubes for 1 hour on each of 2 successive days.

From the stocks of each strain six working cultures were prepared on yeast extract mannitol agar slants (Vincent, 1970). Slants were stored in a refrigerator at 4°C after approximately 7 days of incubation at room temperature. The working cultures were used as standards over a period of 1 year without additional transfers.

Inoculum for soil was prepared by transferring a loopful of culture from a working culture to 20 ml yeast extract mannitol broth (Vincent, 1970). The broth cultures were cultured in an incubator shaker at 29°C for 7 days at which time the cultures were in the late log phase of growth. The cell densities of strain Tha-201 and TAL-309 used for inoculating non-sterilized soil were respectively, 5.6×10^8 and 7.0×10^8 cells ml^{-1}. The cell densities of strains Tha-201, TAL-309, and T-1 used for inoculating sterilized soil were respectively, 5.7×10^8, 5.5×10^8 and 6.7×10^8 cells ml^{-1}. Soil was inoculated by adding 0.5 ml of broth culture to 10 g samples of soil contained in 15 x 100 mm test tubes with plastic caps. The appropriate quantity of sterile dis-

tilled water was added to adjust the soil to -0.33 bar moisture. For the dry treatment, caps were removed from the tubes and the soil was air dried in a laminar flow hood at room temperature for 2 hours before replacing caps and placing the tubes in an incubator. Tubes containing moist soil were kept in a moist chamber to reduce moisture loss. Non-sterilized soil was incubated at 45°C for 15 days but sterilized soil was incubated at 40°C for 45 days.

Two replicates of non-sterilized soil were used for isolation of rhizobia immediately after inoculation and again 15 days after inoculation. The entire 10 g samples were added to 90 ml of sterile distilled water contained in screw-cap bottles and mechanically shaken for 5 minutes. Peanut (Arachis hypogaea L.) seeds were surfaced sterilized with a 10% solution of commercial household bleach for 5 minutes and rinsed several times with tap water (60°C) to remove all traces of the bleach. Four sterilized seeds were sown in modified Leonard jars containing vermiculite. The seeds were inoculated with a soil suspension of Tha-201 by dribbling the entire sample onto the seeds. After inoculation seeds were covered with vermiculite. Seedlings were thinned to two plants per pot. For TAL-309, the same procedure was employed except that cowpea (Vigna unguiculata (L.) Walp) was the host plant. Uninoculated soil was used for inoculation of peanut and cowpea in some pots to provide a check for indigenous rhizobia.

Plants used for isolating rhizobia were supplied with ¼ strength plant nutrient solution (Munns, 1968) except for Mg which was used at ½ strength and were placed in a light room providing a photon flux density of 650 uE $m^{-2}s^{-1}$ at the top of the plants. The temperature of the room was maintained at 29°C during a 14 hour light period and at 21°C during 10 hours of dark each day. At 5 weeks of plant growth all nodules were collected and more than 40 nodules from plants in each container were used for isolation of rhizobia. Nodules were surface sterilized by first rinsing in 95% ethanol and then soaking in 10% bleach for 3 to 4 minutes. The nodules were rinsed repeatedly in sterile water to remove residual bleach. Rhizobia were isolated by piercing a nodule with a flamed, 24 gauge, needle and streaking on YMA (yeast extract mannitol agar) containing congo red. After 7 to 10 days colonies characteristic of rhizobia were transferred to agar slants.

A total of 40 isolates were obtained from each of the two replicates. After culturing on slants for approximately 1 week the cultures were stored in a refrigerator at 4°C.

Immediately following inoculation of sterilized soil and 45 days after inoculation YMA spread plates were utilized to obtain single colony isolates. After incubation of plates for 10 days, 30 colonies of each strain were transferred to slants. Slants were stored in a refrigerator at 4°C after culturing. Isolates obtained from nodules and sterilized soil were confirmed as the isolates of the inoculum strains by using the fluorescent antibody technique (Schmidt et al, 1968).

For effectiveness assays a loopful of rhizobia was aseptically transferred from YMA slants to 6 ml of YMB (yeast extract mannitol broth) in 15 x 100 mm culture tubes having plastic closures. Tubes were placed in test tube racks and were shaken on an incubator shaker at 29°C for 5 days. The populations of rhizobia in the broth after 5 days ranged between approximately 1×10^7 to 10^8 cells ml^{-1}. Six siratro (Macroptilium atropurpureum (DC.) Urp) seedlings in separate compartments of plastic pouches were inoculated singly with each isolate at a rate of 1 ml broth per plant. The plants were grown in the same light room as those used for nodule isolations. Because of labor and space restrictions all treatments could not be tested at the same time. To compensate for this procedure 12 to 24 plants inoculated with a working culture were included in each test so that plant growth conditions over time could be compared.

The quantity of dry matter produced in plants top after 5 weeks of growth was used to group plants into 12 categories. Categories were in 10 mg increments beginning with 20-30 mg. Plant dry weights of parent cultures and uninoculated controls were not included in the analysis of variance. The variances of subpopulations within each date of sampling were analyzed using the SAS (Statistical Analysis System) computer program. Homogeneity of variance between treatments was tested by the F-max ratio method which is the ratio of the largest to the smallest treatment variances (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Stability of effectiveness of cowpea rhizobia in non-sterilized soil was investigated using plant dry weight as the effectiveness characteristic. Since N free plant nutrient solution was utilized the quantity of N fixed would be expected to be highly correlated with plant dry weight (Erdman and Means, 1952, Wynne et al., 1980, and Haydock et al., 1980). Plants used for effectiveness assays of rhizobial isolates from different treatments were not grown at the same time but in the same controlled environment. Inoculation of some plants, in each assay, with parent stock cultures provided controls for comparing growth conditions between treatments. Growth conditions for treatments using non-sterilized soil were not significantly different (Table 1).

Incubation of TAL-309 in non-sterilized soil for 15 days greatly increased the variability of effectiveness in the surviving population (Fig. 1). Square root or logarithmic transformations of data did not result in homogeneity of variance. The mean effectiveness of the populations following incubation were less effective than the parent culture and the mean effectiveness of the 0 day population (Fig. 1). Approximately 37% of the isolates from samples incubated for 15 days produced plants with dry weights less than 60% of the parent cultures whereas none of the isolates from the 0 day treatment were so ineffective. Of the isolates from the moist soil treatment 8% were classified as ineffective (Fig. 1) since the plants were yellowish in color and dry weights were similar to uninoculated plants.

Variances for treatments of strain Tha-201 in non-sterilized soil were not significantly different (Fig. 1). Incubation of this strain in non-sterilized soil did not increase the variability of effectiveness in the population (Fig. 1).

The populations of strains TAL-309 and Tha-201 incubated in dry and moist soil for 15 days at 45°C were decreased by approximately 1000 times indicating pressure against survival was applied. The increased variation in effectiveness of the TAL-309 population indicates that selective pressures were also acting on the effectiveness trait.

Confounding, in the first experiment, due to the possibility of plant selectivity during nodulation occurred since isolates were obtained from nodulated roots and not directly from soil. In a second experiment sterilized soil was used so that rhizobia could be isolated directly from soil by plating. Because of reduced labor requirements an additional strain T-1 was included. The incubation time was increased to 45 days because reduced stress from removal of biotic factors and lowering incubation temperature from 45°C to 40°C increased the time needed for selective pressures on survival (Boonkerd and Weaver, 1982). Populations of strains in moist soil did not decrease in 45 days but in the dry soil the populations decreased by a factor of 10, 1000 and 100 for strains Tha-201, T-1 and TAL-309 respectively (Boonkerd and Weaver, 1982).

Plant growth conditions were not consistently the same during the effectiveness assays of isolates obtained from the soil (Table 1) and must be considered when making treatment comparisons. The mean effectiveness of the populations immediately following inoculation and after incubation for 45 days in moist soil were similar to their respective controls (Fig. 2). The variances of populations for these two treatments were only different for TAL-309 in which case the variance was reduced.

Exposure to dry soil conditions for the 45 day period resulted in treatment affects on all strains (Fig. 2). Variance for strain Tha-201 was increased but the population mean was similar to the parent culture mean. Variances for the other two strains were similar to the respective preincubation treatments. The mean effectiveness of the populations was probably significantly lower than the respective parent cultures considering the magnitude of the differences and the low standard errors of the sample means (± 2) and the standard errors of the parent culture means (Table 1). For TAL-309 the dry treatment resulted in a statistically significant (.05 level) reduced mean effectiveness as compared to the 0 day treatment.

Inspection of the data in Figures 1 and 2 of day 0 treatments of both experiments reveals that the shape of the frequency distributions for effectiveness of respective strains were very similar. Apparently

there was no detectable difference due to sampling of the soil population for effectiveness by inoculating plants and isolating rhizobia from the nodules or by isolating rhizobia directly from soil by plating.

The results of this research clearly indicate the importance of developing further understanding on the ecology of rhizobia in soil to include stability of symbiotic character for nitrogen fixation. An incubation temperature increase from 40°C to 45°C resulted in a change of 5% to 26% of the isolates of TAL-309 becoming only 60% as effective as the parent culture. Other strains were more stable but some isolates of low effectiveness (less than 60% as effective as the parent culture) were obtained for all strains. Additional research should be directed towards determining the relative abilities of the ineffective isolates to survive in soil. Also it would be beneficial to determine if the relative instability of TAL-309 is due to plasmids that control effectiveness. Certainly it is important for inoculant producers to utilize strains of rhizobia known to have symbiotic characteristics that will be stable in the environments where the inoculum will be utilized.

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Table 1. Effectiveness of the parent cultures used as controls for each treatment for the purpose of determining similarity of growth conditions between assays.

Treatment		Strain TAL-309	Tha-201	T-1
		----------dry weight mg plant^{-1}----------		
Soil				
nonsterile	control	110 (±3.4)[†]	114 (±6.2)	
	dry	111 (±4.3)	100 (±6.3)	
	moist	118 (±5.1)	112 (±7.2)	
		N.S.	N.S.	
Soil				
sterilized	control	114 (±11.6) a[*]	118 (±6.9) a	98 (±3.3) b
	dry	116 (± 5.1) a	110 (±5.1) a	113 (±4.8) a
	moist	92 (± 4.7) b	82 (±3.2) b	86 (±3.8) b

[†]Number in parenthesis is the standard error of the mean. For nonsterile soil treatments averages are of 21 to 24 plants and for sterilized soil treatments averages are of 11 plants.

[*]Averages within strains for nonsterile soil were not significantly different at 0.05 probability level by the F. test. For sterilized soil treatments averages having a letter in common are not significantly different by Duncan's new multiple range test.

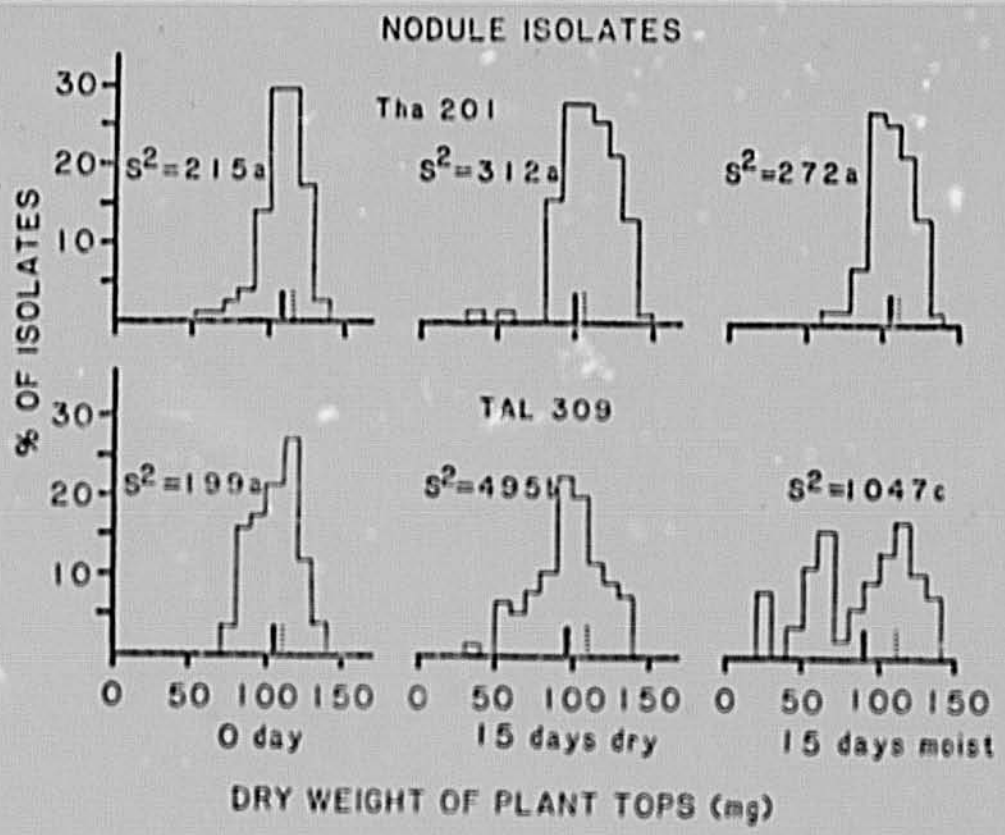

Figure 1. Effectiveness of rhizobial isolates obtained from nodules of plants inoculated with nonsterile soil immediately following inoculation (0 day) with two strains of rhizobia and after 15 days incubation under dry or moist conditions. Each figure is based on 80 nodule isolates. The variance (s^2) of each population is indicated. The variances for treatments of individual strains are not significantly different (α = 0.05) if they have a letter in common. The bars on the abscissa indicate the relative effectiveness of the parent culture (broken) and the mean of the 80 isolates (solid).

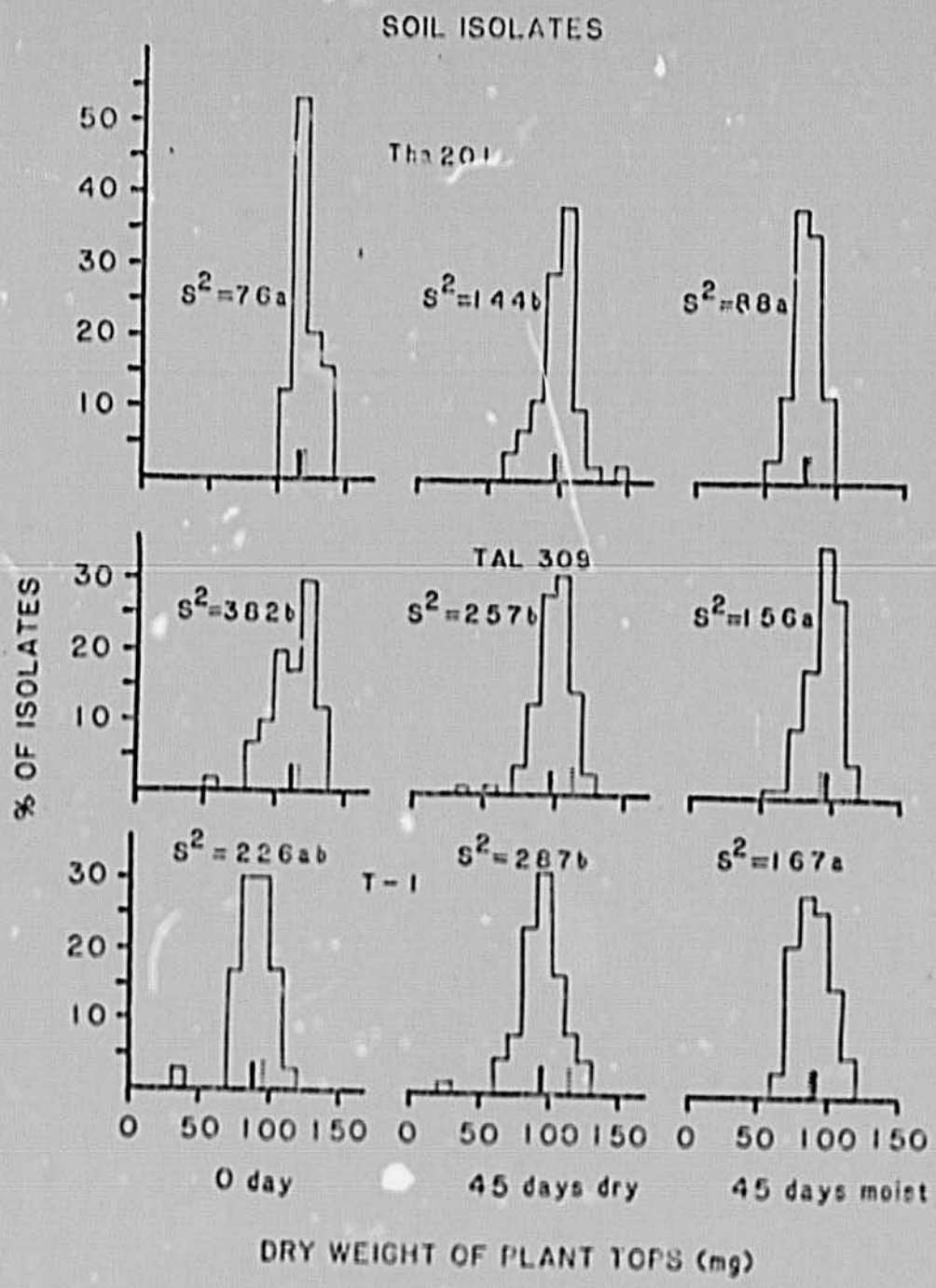

Figure 2. Effectiveness of rhizobial isolates obtained from sterilized soil immediately following inoculation (0 day) with three strains of rhizobia and after 45 days incubation under dry or moist conditions. Each figure is based on 60 isolates obtained by plating soil on yeast extract mannitol agar. See caption to Figure 1 for additional information.

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Survival of Cowpea Rhizobia in Soil as Affected by Soil Temperature and Moisture

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Successful inoculation of peanuts and cowpeas depends on the survival of rhizobia in soils which fluctuate between wide temperature and moisture extremes. Survival of two cowpea rhizobial strains (TAL309 and 3281) and two peanut rhizobial strains (T-1 and 201) was measured in two soils under three moisture conditions (air-dry, moist (−0.33 bar), and saturated soil) and at two temperatures (25 and 35°C) when soil was not sterilized and at 40°C when soil was sterilized. Populations of rhizobia were measured periodically for 45 days. The results in nonsterilized soil indicated that strain 201 survived relatively well under all environmental conditions. The 35°C temperature in conjunction with the air-dry or saturated soil was the most detrimental to survival. At this temperature, the numbers of strains T-1, TAL309, and 3281 decreased about 2 logs in dry soil and 2.5 logs in saturated soil during 45 days of incubation. In sterilized soil, the populations of all strains in moist soil increased during the first 2 weeks, but decreased rapidly when incubated under dry conditions. The populations did not decline under saturated soil conditions. From these results it appears that rhizobial strains to be used for inoculant production should be screened under simulated field conditions for enhanced survival before their selection for commercial inoculant production.

The benefits from inoculation depend on survival of introduced rhizobia in the soil. They must survive long enough after sowing to nodulate the host, and it would be desirable for the rhizobia to persist between cropping seasons. Soil moisture content and temperature are factors that strongly influence survival of rhizobia. In a field environment soil moisture content and temperature are dynamic and fluctuate between wide ranges. Many countries have distinct wet and dry seasons; soil temperatures fluctuate in subtropical climates, but remain relatively high all year in tropical countries. Cowpeas and peanuts are economically important food legumes that are nodulated by rhizobia in the cowpea cross-inoculation group. These legumes are adapted to subtropical and tropical regions.

The selection of rhizobia for inoculants should include evaluations of their ability to survive in soil (2). Published data indicating the importance of soil moisture, temperature, and interactions between these factors on survival of cowpea rhizobia are very limited. *Rhizobium* as a genus is sensitive to low soil moisture conditions. Survival in soil maintained at low water tension (near field capacity) is much superior to survival under conditions of high water tension (3, 5, 8). Generally the population declines by 2 logs within a few days when a moist soil undergoes drying at moderate temperature (3, 8). Evidence indicating consistent differential sensitivity of fast- and slow-growing rhizobia is not conclusive (1, 3, 8). Strains of cowpea rhizobia differ in their susceptibility to desiccation in silica sand at 27°C (3). Populations of some strains declined by 1 log in 3 weeks, whereas the populations of other strains declined by 3 logs.

Data are not available on survival of cowpea rhizobia in soil maintained under moisture and temperature conditions frequently encountered in field environments. Because of the limited amount of information on survival of cowpea rhizobia and their importance in tropical and subtropical agriculture, experiments were undertaken in our laboratory to evaluate the effect of soil temperature, moisture, and their interaction on survival of cowpea rhizobia.

MATERIALS AND METHODS

Soils. The survival of *Rhizobium* in the cowpea cross-inoculation group was investigated by using two soils. A Korat sandy loam was obtained from Thailand by air freight and is a ustoxic dystropepts with a pH of 5.7 in a 2:1 (w/w) water-soil suspension. The other soil, a Lufkin sandy clay loam, was obtained in Texas and is a Vertic Albaquaff with a pH of 6.8. Both soils are well suited to cowpea production, but only the soil from Thailand is commonly used for peanut produc-

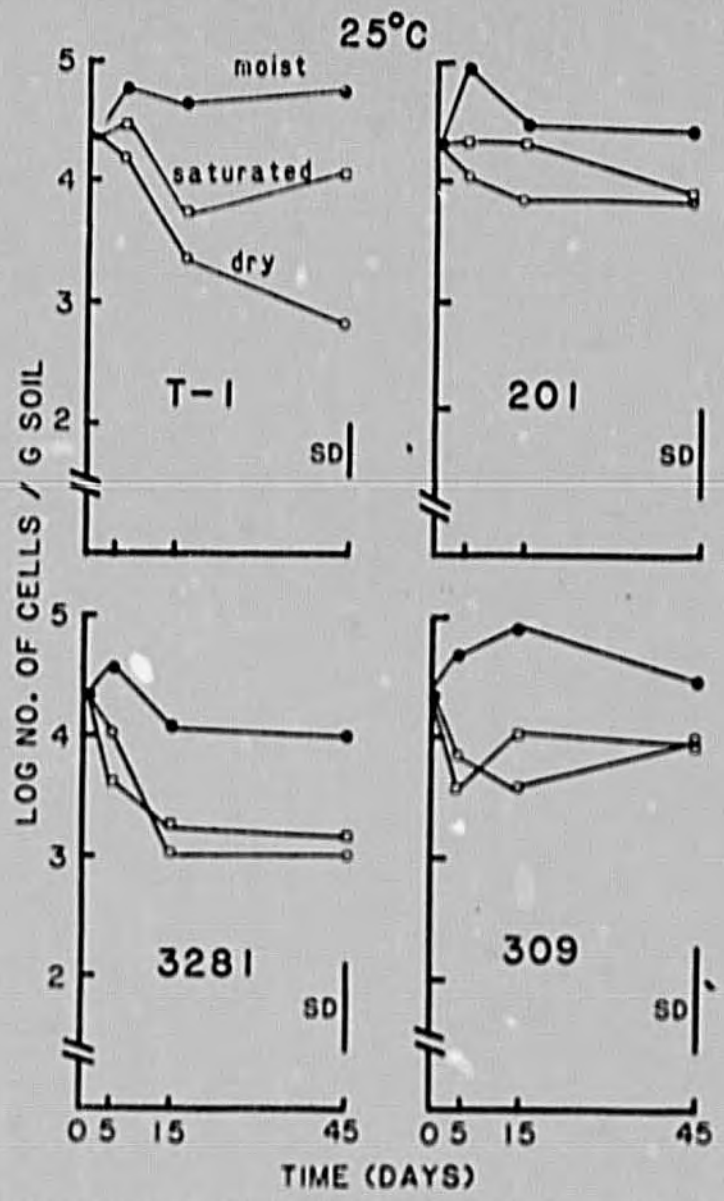

FIG. 1. Survival of cowpea rhizobia strains in nonsterilized dry, moist, and saturated soil incubated at 25°C.

tion. The particular areas from which soil was collected were not known to have been planted to cowpeas or peanuts. Siratro (*Macroptilium atropurpureum*) was grown on samples of soil and did not nodulate, indicating the absence of cowpea rhizobia.

Strains and inoculum. Two strains of *Rhizobium*, TAL309 and 3281, which nodulate cowpeas were utilized in the investigations and were respectively obtained from the NifTAL culture collection (Paia, Hawaii) and the U.S. Department of Agriculture culture collection (Beltsville, Md.). Two other strains, 201 and T-1, were respectively isolated from field-grown peanuts in Thailand and Texas. Inoculum was prepared by growing rhizobia in yeast extract-mannitol broth (10) to a cell density of approximately 4×10^8 cells per ml, which corresponded to the late log phase of growth.

Inoculation. Each strain of *Rhizobium* was inoculated into 10-g samples of air-dry soil contained in test tubes (15 by 100 mm) with plastic caps. Inoculation of sterilized soil was accomplished by adding 0.5 ml of broth culture to each tube. For nonsterilized soil a 1:10 dilution of broth was made with 0.1% peptone, and 0.5 ml was used as the inoculum. Moisture was distributed by shaking individual tubes by hand until the moisture appeared evenly distributed as was apparent by uniform soil color.

Moisture treatments. Distilled water was added after inoculation to prepare the moisture treatments: −0.33 bar moisture tension and flooded (1-cm water layer

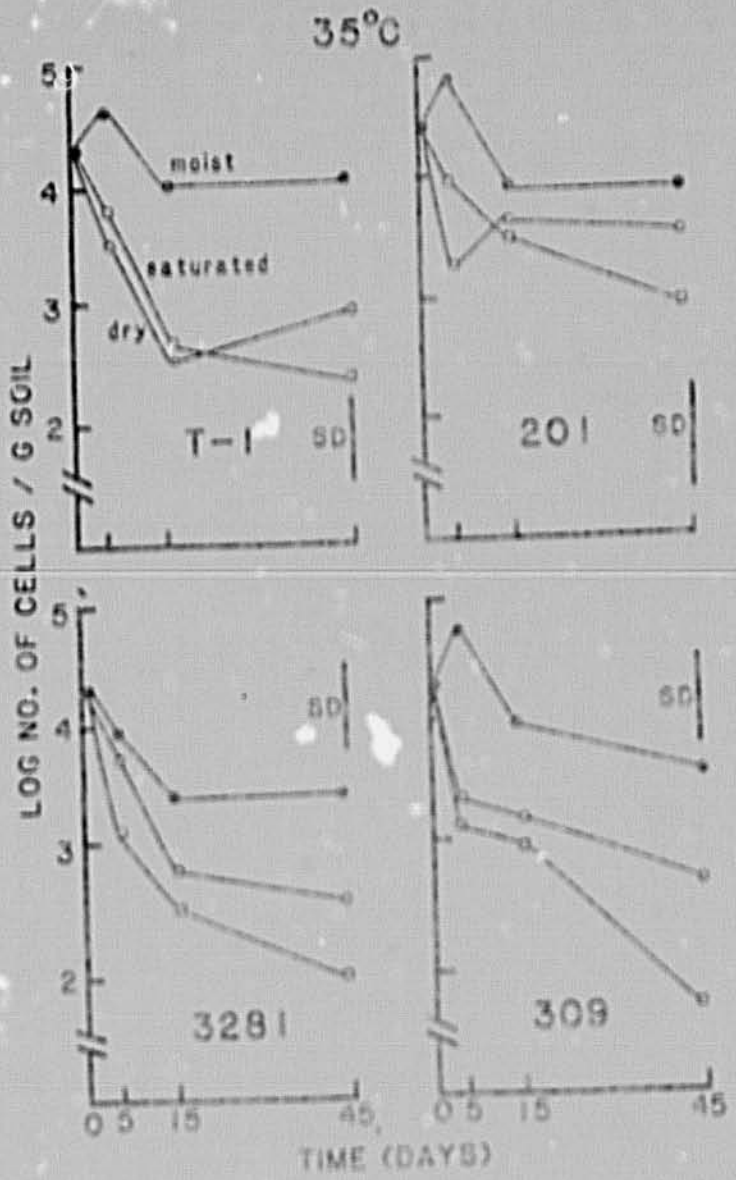

FIG. 2. Survival of cowpea rhizobia strains in nonsterilized dry, moist, and saturated soil incubated at 35°C.

over soil surface). A pressure membrane apparatus was used to determine soil moisture content at −0.33 bar moisture tension. The dry treatment was achieved by placing inoculated samples, with caps removed, in a laminar flow hood for 2 h after which the soil appeared dry. The water content of moist samples was maintained during incubation by keeping them in a plastic humidity chamber lined with moist paper towels. Periodic weighing of samples indicated insignificant moisture loss. The samples for the dry treatment were exposed directly to the air in the incubator.

Soil sterilization. Soil was sterilized by autoclaving the 10-g samples in test tubes for 1 h on each of 2 successive days.

Enumeration of rhizobia. Rhizobia inoculated into nonsterilized soils were enumerated by the plant probable number method (11). Soybean was the indicator plant and was examined for nodulation 3 weeks after inoculation. Four replications and 10-fold serial dilutions in plant nutrient solution were used in the most probable number procedure. Rhizobia inoculated into sterilized soil were enumerated by making 10-fold dilutions in 0.1% peptone and spread plating 0.1 ml on yeast extract mannitol agar (10). Cultures were incubated for 10 days at 29°C before counting. Two soil samples of each treatment were processed at each sampling, and the entire 10-g samples were utilized.

RESULTS AND DISCUSSION

Survival of rhizobia in the two nonsterilized soils was not significantly different ($\alpha = 0.10$).

TABLE 1. Survival of cowpea rhizobial strains in sterilized soil incubated at different moistures at 40°C

Strain	Moisture	Survival[a] after incubation time (days):			
		0	5	15	45
201	Dry	7.53	6.53	6.25	6.41
	Moist	7.53	8.15	8.20	8.17
	Saturated	7.53	7.32	7.28	7.92
T-1	Dry	7.53	4.99	5.00	4.30
	Moist	7.53	8.15	8.20	8.17
	Saturated	7.53	7.32	7.28	7.92
TAL309	Dry	7.25	6.32	6.28	5.43
	Moist	7.25	7.30	7.66	7.36
	Saturated	7.25	6.80	6.83	6.84

[a] Data are presented as log units per gram of soil; standard deviation, ±0.32.

and there was no significant ($\alpha = 0.10$) interaction of soil types with other factors. For brevity, only averages for the two soils are presented.

Inspection of the data for rhizobial survival at 25°C (Fig. 1) reveals that strains 201 and TAL309 maintained relatively high populations for 45 days under all moisture conditions. Strain T-1 withstood saturated soil conditions better than dry soil conditions (Fig. 1). Survival of strain 3281 under saturated and dry soil conditions was similar and relatively poor as compared with survival under moist conditions (Fig. 1).

Incubation of rhizobia at 35°C was more detrimental than at 25°C, but again strain 201 survived well relative to the other strains (Fig. 2). For all strains the tendency was to have more viable rhizobia present at 45 days in soil maintained under dry conditions than under saturated conditions (Fig. 2). The standard deviation was too large for statistical significance between these moisture treatments for individual strains, but combined analysis of variance indicated that the saturated conditions were significantly ($\alpha = 0.05$) more detrimental than the dry conditions. The contrast in survival of strain TAL309 between the different moisture treatments and temperatures (Fig. 1 and 2) indicates the importance of strain evaluations being made for several conditions.

The results obtained with nonsterilized soils provided no opportunity of separating the effects of biotic and abiotic factors on rhizobial survival. A second inoculation experiment was conducted with a sterilized soil to eliminate biotic factors. Strain 3281 was not included to reduce labor requirements, and the incubation temperature was increased to 40°C to increase pressure on survival. Under moist soil conditions rhizobial populations did not decline for 45 days (Table 1). They also survived very well under saturated conditions (Table 1), in contrast to the results with nonsterilized soils (Fig. 1 and 2). The population actually increased for strains 201 and T-1 under saturated soil conditions and only slightly decreased for strain TAL309 (Table 1). Mahler and Wollum (6) also reported that after an initial reduction in the population of *Rhizobium japonicum* in sterilized soil the population increased. Apparently after a period of adjustment rhizobia can reproduce in soil when competitors are eliminated. Under dry moisture conditions the populations declined for all strains (Table 1), but as occurred for the nonsterilized soils (Fig. 1 and 2), strain 201 indicated better survival than the other strains.

The results of this investigation confirm those of Vidor and Miller (9), with *R. japonicum*, that biotic factors are important in controlling rhizobial populations in moist soil exposed to moderate temperatures. Some of the biotic factors that may be responsible for the death of rhizobia in the nonsterilized flooded soil are bacteriophage (9), *Bdellovibrio* (4), and protozoa (7). The population of *Rhizobium trifolii* and *R. japonicum* in sterile and nonsterile soil was not significantly different when soil moisture content was approximately 50% of field capacity, but the decline in population was substantial in nonsterile saturated soil (7). Moreover, the population of indigenous protozoa did not increase appreciably in moist soil, but did increase appreciably in saturated soil and coincided with a rapid decline in the number of rhizobia (7). Survival of cowpea rhizobia in our study was very good in moist nonsterilized soil, which indicates that the population decline in saturated nonsterilized soil was more likely due to protozoa than to bacteriophage or *Bdellovibrio*. If this soil contained bacteriophage or *Bdellovibrio* the rhizobial populations in nonsterile moist soil would also have been expected to be reduced (7).

Strains of rhizobia are commonly selected for inoculant production based solely on their sym-

biotic capacity to fix nitrogen. Date (2) emphasized the need for inoculum strains to have saprophytic competence. Our results clearly demonstrate that strains of cowpea rhizobia have different capacities for survival in soil exposed to moisture and temperature conditions that commonly occur both in tropical and temperate regions. Temperature and moisture interacted in influencing survival of strains, which indicates that rhizobia should be tested for survival at more than one temperature and moisture content. Because survival of rhizobia in nonsterilized soils was different than in sterilized soils, it appears that survival tests should be conducted with nonsterilized soils even though enumeration of rhizobia under such conditions is more difficult. Data on survival of rhizobia in soil should be considered with information on nitrogen-fixing capacity when recommending new rhizobial strains for inoculants.

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COWPEA RHIZOBIA: COMPARISON OF PLANT INFECTION AND PLATE COUNTS

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Summary—Enumeration of *Rhizobium* in soil is usually accomplished by the most probable number (MPN) plant infection method. The accuracy of MPN counts as compared to plate counts was determined for five strains of cowpea rhizobia. Host plants included cowpea (*Vigna unguiculata* (L.) Walp.), siratro (*Macroptilium atropurpureum* (DC.) Urb.) and peanut (*Arachis hypogaea* L.). Plastic growth pouches were used primarily for cowpea and siratro while plastic cups containing vermiculite were used for peanut. The number of rhizobia determined by the MPN method using cowpea, siratro and peanut underestimated the population from 10- to 100-fold. A control experiment using soybeans (*Glycine max* (L.) Merrill) indicated that the MPN method was accurate for *R. japonicum*. Experimentors using the MPN method should be aware of its accuracy for particular *Rhizobium*-legume combinations.

INTRODUCTION

The plant-infection dilution assay (MPN), is the most frequently used technique to enumerate rhizobia in soil and inoculants when other microorganisms are present. It has proven accurate for counting *Rhizobium japonicum*, *R. meliloti*, *R. trifolii* and *R. leguminosarum* (Date and Vincent, 1962; Brockwell, 1963; Weaver and Frederick, 1972) but the accuracy of the MPN plant-infection method for enumerating cowpea rhizobia has not been established. Date and Vincent (1962) presented evidence, using one cowpea strain of rhizobia, that the method is accurate but Wilson and Trang (1980) reported that the population of cowpea rhizobia in inoculants was grossly underestimated. Wilson and Trang (1980) speculated that a decline in cell vigor during inoculant storage may have caused the underestimation.

While determining survival of cowpea rhizobia in soil, it became apparent to us that populations were underestimated by the MPN plant-infection method. Several other investigators (Herridge and Roughley, 1974; Day *et al.*, 1978; Philpotts, 1977) have used the MPN method to enumerate cowpea rhizobia but they did not report on its accuracy. Our purpose is to present evidence that the MPN plant-infection method is not reliable for cowpea rhizobia and that different indicator plants provided similar results.

MATERIALS AND METHODS

Five strains of cowpea rhizobia were used. Strains Thai-201 and T-1 were isolated by us from field grown peanuts. The other three strains; TAL309, CB756 and 3281 (316b15) were obtained from the Niftal culture collection (Paia, Hawaii). The Nitragin Company (Milwaukee, Wisconsin) and the USDA *Rhizobium* culture collection (Beltsville, Maryland) respectively and were known to be effective on cowpeas. *R. japonicum* strain 110 was obtained from the USDA *Rhizobium* culture collection. Six day-old cultures grown in yeast mannitol broth were used for inoculations.

Different plant hosts were used and included: six varieties of cowpea (*Vigna unguiculata* (L.) Walp.), "Black eye", "Bush", "Calico", "Brawder", "Queen Anne" and "Pink eye", siratro (*Macroptilium atropurpureum* (DC.) Urb.), peanuts (*Arachis hypogaea* L.) var. "Tamnut 74" and soybeans (*Glycine max* (L.) Merrill) var. "Coker 338".

Plastic pouches (Weaver and Frederick, 1972) were primarily used for cowpea, siratro and soybeans. Agar (Vincent, 1970) and vermiculite in small bottles, were also compared with plastic pouches. Plastic drinking cups (250 ml) filled with vermiculite were used for growing peanuts. Seed were germinated and only healthy seedlings planted.

Plate counts of broth cultures were made within 2 h of inoculating plants. Duplicate plates of yeast-extract mannitol agar (Vincent, 1970) were used for plate counts. The diluent for MPN plant-infection counts was N-depleted plant nutrient solution (Munns, 1979) which was also used for watering plants. Ten-fold serial dilutions and four replications per dilution were used for MPN counts. Plants were maintained for at least 3 weeks before considering them as negative (nonnodulating).

The plants were grown in a light room equipped with fluorescent lamps which provided a photon flux density 650 $\mu l\ m^{-2}\ s^{-1}$ at the top of the plants. The temperature of the room was maintained at 29°C during 14 h of light and at 21°C during 10 h darkness.

RESULTS AND DISCUSSION

The populations, of cowpea rhizobia, were measured by MPN plant-infection and plate counts using different strains of cowpea rhizobia and different indicator plants. Using cowpea and siratro as in-

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Table 1. Log_{10} of rhizobia measured by MPN and plate counts using different strains of *Rhizobium* and legumes

Strains	Plate count[a]	MPN[b]-plant infection Soybeans	Cowpea	Siratro
110	8.69	7.90	ND[c]	6.48
110	7.00	6.49	ND	ND
201	8.88	ND	6.46	6.81
309	8.54	ND	6.05	6.64
T-1	8.78	ND	6.49	6.97
3281	8.93	ND	6.49	6.00

[a] Average SD ± 0.03.
[b] Factor for 95% fiducial limits ± 0.58.
[c] ND—Not determined.

Table 3. Comparison of MPN and plate counts using different strains of *Rhizobium* and varieties of cowpeas

Strains	Cowpea varieties	Plate[a] log_{10} No/l	MPN[b] log_{10} No/l
201	Black Eye	8.75	6.49
201	Queen Anne	8.75	6.76
3281	Black Eye	8.95	7.23
3281	Queen Anne	8.95	6.76
3281	Bush	8.95	7.23
3281	Calico	8.95	6.49
309	Black Eye	8.47	7.23
309	Queen Anne	8.47	6.49
309	Bush	8.47	6.76
309	Calico	8.47	6.49
309	Brawder	8.47	7.23
309	Pink eye	8.47	6.76

[a] Average SD ± 0.02.
[b] Factor for 95% fiducial limits ± 0.58.

dicator plants resulted in MPN counts approximately 2 log_{10} units lower than plate counts for all rhizobial strains (Table 1). *R. japonicum* strain 110 and soybean were included as a control and indicated close agreement between counting methods. When siratro was used for the MPN-count of strain 110, the number obtained was 2 log_{10} units lower than the plate count.

Use of plastic pouches was not the cause for the low counts because the results of another experiment in which vermiculite and agar were used as the support media provided similar results (Table 2). The MPN values for T-1 using the different plant support media were similar but for CB756, the MPN obtained from using agar was 1 log_{10} unit higher than for vermiculite and plastic pouches but was 1 log_{10} lower than plate counts.

To be certain the low MPN counts were not due to the particular variety of cowpea, five additional varieties were used as indicator plants. The results indicated the MPN's obtained from using different varieties were not significantly different and populations of all rhizobial strains were underestimated (Table 3).

Peanut was used as a third plant nodulated by cowpea rhizobia. Peanuts were grown for MPN counts in 250 ml cups containing vermiculite because healthy root systems do not always develop when peanuts are grown in plastic pouches. Plate and MPN counts of strains T-1 and 201 were made. The plate counts were respectively 7.66 and 7.43 and the MPN's were respectively 6.49 and 5.49 log_{10} units. Both strains were highly effective on peanuts (Weaver, 1974; Sen and Weaver, 1981). Brockwell (1963) reported that when plants were grown in vermiculite, the MPN underestimated the population of *R. trifolii* by approximately 40%.

The MPN method using plant infection to enumerate cowpea rhizobia was found to be inaccurate. The results indicate the basic assumption, implicit in the use of the MPN plant-infection method, that a single *Rhizobium* cell can multiply and result in nodule formation on cowpea and siratro is invalid. Wilson and Trang (1980) using the MPN plant-infection method to enumerate cowpea rhizobia in inoculants also reported that the MPN count was lower than the plate count. They speculated that the lower MPN might be due to loss of infectiveness of some cells during storage or to poor survival in the plant nutrient solution after inoculation of seedlings. We used more than 100 single colony isolates from plates to inoculate cowpeas and without exception all formed nodules on the plant indicating no loss of infectiveness. The rhizobia not only were capable of surviving in the plant nutrient solution but the population increased with time (Table 4).

We found that prolonging the time for growing plants from 3 to 6 weeks after inoculation did not increase the accuracy of the method because the roots of plants grown in plastic pouches and in small containers do not remain vigorous and healthy beyond 4 weeks. Inoculation of seedlings of peanuts, cowpeas or siratro with large populations of rhizobia resulted in nodulation within 7–10 days. From these considerations it appears that 3 weeks is a reasonable period to expect responsive plants to nodulate.

The results obtained for rhizobial strains used in our investigations may not be representative for other strains. Nevertheless, since the method was inaccurate for some strains, investigators should be cautious about using it to enumerate cowpea rhizobia. When it

Table 2. Log_{10} number of rhizobia measured by MPN plant-infection using siratro grown in different media as compared to plate counts

Strains	Plate count[a]	MPN[b]-plant infection Vermiculite	Pouches	Agar
T-1	8.27	6.76	6.76	7.00
CB756	7.57	5.23	5.49	6.49

[a] Average SD ± 0.02.
[b] Factor for 95% fiducial limits ± 0.58.

Table 4. Growth of cowpea rhizobial strain T-1 in sterile nutrient solution and 0.1% peptone

Days	Nutrient solution (cells ml⁻¹)	Peptone (cells ml⁻¹)
0	70	70
1	71	93
2	200	217
3	732	1200

is used, evidence should be provided that it is accurate for the particular rhizobial strains being used. Future research needs to be undertaken to determine why the population estimates of cowpea rhizobia using the MPN plant-infection method were so low.

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